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INCIDENCE, ECOLOGY, AND FATE OF TARGET FOODBORNE PATHOGENS IN
THE CHEESEMAKING CONTINUUM

A Dissertation Presented

by

Dennis D'Amico

to

The Faculty of the Graduate College

of


The University of Vermont


In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Animal, Nutrition and Food Sciences

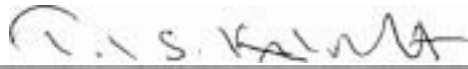
October 2008


Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Animal, Nutrition and Food Sciences

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Abstract

Due to renewed interest in specialty cheeses, small-scale artisan and farmstead producers are manufacturing numerous varieties of cheese, including those that present higher risk such as surface-ripened soft cheeses, often using raw milk. The presence of pathogenic bacteria in raw milk on large scale dairy farms is well documented as the dairy farm itself can serve as a reservoir. To assess the risks associated with the use of raw milk in the manufacture of small-scale artisan cheese we evaluated overall milk quality and prevalence of four target pathogens (*Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp., and *Escherichia coli* O157:H7) in raw milk from 11 farmstead cheese operations in Vermont. Although overall incidence was low in comparison to other surveys, variation from farm to farm, independent of species, indicates that some operations practice strict hygienic controls and that additional effort is needed on others. Our data also suggest that if present, pathogen population levels in raw milk are extremely low. Although these pathogens are readily inactivated by pasteurization, pasteurized milk and milk products, including soft cheese, have been implicated in major outbreaks of *L. monocytogenes* infection as the result of post-processing environmental contamination. U.S. Standards of Identity permit the manufacture of these and other cheeses from raw milk, provided they are aged for a minimum of 60 days typically at a temperature no less than 35°F (1.67°C) to provide safety. Of particular concern are the surface-mold-ripened soft cheeses due to the growth potential during aging and refrigerated holding following increases in pH. In a study on the growth potential of *L. monocytogenes* introduced as post process contaminants on surface-mold-ripened cheeses we demonstrate that holding cheese in compliance with U.S. Federal regulations supports pathogen growth from very low levels regardless of the whether the milk used was raw or pasteurized. Moreover, the 60 day aging rule encourages extended holding which could inadvertently contribute to risk. It is clear that the safety of cheeses within this category must be achieved through control strategies other than pasteurization or aging. Effective environmental monitoring and control of *Listeria* spp. within processing plants, including farmstead cheese operations, is considered paramount in reducing cross contamination of ready-to-eat foods. To assess the incidence and ecology of *Listeria* spp. in farmstead cheese processing environments we conducted environmental sampling in 9 different cheesemaking facilities over a 10-week period while comparing three detection/isolation protocols. Results indicate that the use of detection/isolation methods incorporating dual primary enrichment with a repair step allowing for the recovery of injured *Listeria* enhances detection of *Listeria* spp., including *L. monocytogenes*, and that the addition of PCR increases sensitivity of detection while greatly reducing time to results. Our data indicate that the extent of farmstead cheese plant contamination with *Listeria* spp., notably *L. monocytogenes*, is comparatively low for dairy processing plants, especially those with contiguous farms and aids in the identification of control points for use in designing more effective control and monitoring programs. Overall the studies contained herein fill data gaps in the literature considering the threat of emerging pathogens in raw milk intended for farmstead cheesemaking, as well as the incidence and distribution of *Listeria* spp., including molecular subtypes, in small farmstead cheese processing environments over time. These data will help inform risk assessments which evaluate the microbiological safety of artisan cheeses, particularly those manufactured on farms.

Citations

Material from this dissertation has been accepted for publication in the Journal of Food Protection on March, 3rd, 2008, in the following form:

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Comprehensive Literature Review

United States Regulatory Policy Regarding Milk and Milk Products

Regulation of milk

Concerns regarding the safety of milk began in the mid-1800s as consumption of fluid milk increased, primarily in big cities (Block, 2002). Prior to World War II, poor sanitation, improper milk handling and animal health issues resulted in numerous outbreaks of diphtheria, brucellosis, tuberculosis and typhoid fever from the consumption of unpasteurized milk (Ryser, 2001). Although many large cities began to mandate the pasteurization of all milk for sale at this time, without a standard definition of the process as exists today under regulation, treatments may have only eliminated spoilage organisms and other natural milk flora, as opposed to pathogens (Johnson et al, 1990).

In 1924 the United States (U.S.) Public Health Service developed the Standard Milk Ordinance as a model regulation for voluntary adoption by State and Local Milk Control Agencies to control milkborne disease. Known today as the Grade "A" Pasteurized Milk Ordinance (PMO), this document governs the processing, packaging, and sale of Grade "A" milk and milk products (USFDA, 2004b). The PMO specifically describes methods recommended for proper production, transportation, processing, handling, sampling, examination, and labeling of dairy products, including guidelines for the inspection of dairy farms and processing plants (USFDA, 2004b). Regulations regarding the methods of pasteurization are very specific in regards to time-temperature applications and equivalent processing standards assuring the safety of dairy products. While standard pasteurization parameters were most recently revised in the 1950s to

inactivate the highly heat stable pathogen *Coxiella burnetti*, they remain effective against all recognized human pathogens. With the gradual implementation of pasteurization, coupled with improved milk quality, herd management and milk collection practices (Johnson et al. 1990), the risk of milkborne infection has decreased significantly (CDC, 1999) with no reports of milkborne diphtheria, scarlet fever, tuberculosis or typhoid fever in over 40 years (Ryser, 2001). As a testament to its efficacy, milk and fluid milk products are currently associated with less than four percent (3.6%) of all disease outbreaks due to contaminated foods (DeWaal and Bhuiya, 2007), compared to twenty-five percent (25%) in 1938 (USFDA, 2004b).

Beginning in the 1970's, changes in agricultural practices and food processing operations, and the globalization of the food supply have resulted in the emergence of newly recognized foodborne pathogens in the United States (CDC, 1999) including *Listeria monocytogenes*, multi-drug resistant (MDR) *Salmonella* species, *Campylobacter jejuni/coli* and shiga-toxin producing strains of *Escherichia coli* (STEC). From 1973 to 1992, 46 raw milk associated foodborne disease outbreaks resulting in 1,733 illnesses were reported to the Centers for Disease Control and Prevention (CDC) with *Campylobacter* and *Salmonella* species constituting a majority of cases (Headrick, 1998). In 1987, unpasteurized milk and milk products in final package form intended for human consumption were banned from interstate commerce (21 CFR Section 1240.61). Although these products are still permitted for intrastate sale in some states, the mean annual number of reported outbreaks attributed to milk decreased dramatically following the ban (Headrick, 1998). While the Grade "A" PMO provides guidance to ensure that

fluid milk is produced safely, milk intended for cheesemaking is subject to different regulations determined by individual states and not necessarily governed by the PMO (Zeng et al. 2007).

Regulation of cheese

As previously discussed, milk has not always been of the same microbiological quality we enjoy today. According to a review by Johnson and others (1990) the heat treatment of milk destined for cheesemaking was primarily focused on improving the quality and uniformity of cheese prior to the 1950s with researchers in the U.S. and abroad examining the effects of various heat treatments on the overall taste and quality of cheese. In addition to improved quality, it was noted that heat treatment gave cheesemakers improved control over the cheesemaking process and was shown to destroy pathogenic bacteria to varying degrees (Johnson et al. 1990).

During World War II, when millions of pounds of Cheddar cheese were supplied to the Allies and U.S. Armed Forces, the U.S. Department of Agriculture (USDA) encouraged producers to use pasteurized milk as a means of controlling quality (Johnson et al. 1990). However, following two outbreaks of typhoid fever in 1944, possibly acquired from the consumption of cheese, the Surgeon General suggested that cheese be made from pasteurized milk or adequately cured to reduce the risk of illness (Johnson et al. 1990). Soon several states enacted legislation to help ensure the safety of cheese and in 1947, with safety and quality in mind, the U.S. Food and Drug Administration (FDA) held a hearing to discuss newly proposed standards for several cheese varieties (Johnson et al. 1990). As a result of these hearings it was established that pathogenic

microorganisms that may be found in raw milk, shed directly from infected animals or contaminated during handling, are inactivated by pasteurization but not necessarily by temperatures encountered during cheesemaking (Johnson et al. 1990).

The elimination of pathogens in cheese through curing is believed to have been derived from the conclusions of a study detailing the survival of *Brucella abortus* in Cheddar cheese published by Gilman and colleagues in 1946 (Boor, 2005). In their conclusion the authors state that “Cheddar cheese has not been proved to be a carrier for undulant fever and that reported typhoid fever epidemics have not been attributed to cheese cured for more than 63 days” (Gilman et al. 1946). However, in this study *B. abortus* survived up to six months of aging, depending on initial inoculation level. Despite the observed results, it was recommended that 60 days of aging would provide reasonable protection from *B. abortus* in Cheddar (Boor, 2005; Gilman et al. 1946). According to Johnson and colleagues (1990) it was deemed unreasonable at this time to require the holding of cheese for a period long enough to ensure the death of all pathogens as the exact duration of curing necessary to assure safe cheese was unknown. It was, however, deemed reasonable to expect that cheese held for at least 60 days at temperatures no less than 35°C would be safe (Johnson et al. 1990).

The final regulations governing the use of raw milk for cheesemaking promulgated in 1949, require cheesemakers to either pasteurize milk for cheesemaking (phosphatase destruction; 62.8°C for at least 30 min or 71.1°C for 15 sec), or cure the cheese for a specified amount of time as defined by its specific Standard of Identity (21 CFR 133; USFDA, 2003), typically no less than 60 days at a temperature greater than

35°F (1.67°C). Currently, more than 30 natural cheese varieties can be legally made from raw milk in the U.S. provided that they are sufficiently aged (USFDA, 2003).

Challenges to the 60 day aging rule

For decades studies have examined the survival of pathogenic microorganisms in cheese during aging. Most of the early research was focused on Cheddar cheese, likely due to its production volume at that time. According to a review by Marth (1963), the survival of pathogenic bacteria including *Salmonella typhosa* and *Streptococcus pyogenes* in Cheddar cheese beyond 60 days was documented in the early 1940's, prior to the institution of the specific curing requirements for cheese. According to Boor (2005), researchers Van Slyke and Price reported on the extended survival of *Mycobacterium tuberculosis* (>100 days), *Salmonella typhoid* (3-10 months), and hemolytic streptococcus (>160 days) in Cheddar cheese in 1949 (Boor, 2005). Similarly, challenge studies conducted in the 1960s also detailed the survival of pathogens such as *Salmonella typhimurium* beyond the 60 day curing period (Goepfert et al. 1968) while *Salmonella typhi* inoculated into cheesemilk was shown to survive in stirred curd granular Cheddar cheese for 150-180 days when held at refrigeration temperatures (0°C and 4°C; Hargrove et al. 1969).

Despite evidence of the inadequacy of the 60 day aging rule, it remained unchallenged by regulatory bodies. However, in the 1990s, research published in the scientific literature detailed the survival of the emerging pathogen *Escherichia coli* O157:H7 well beyond the mandatory 60 day holding period in Cheddar cheese (Reitsma and Henning, 1996; Shlessor et al. 2006). Despite the limited epidemiological evidence

that foodborne illness results from consumption of raw milk hard cheeses that have been aged for 60 days, the FDA made the reevaluation of the 60 day rule a top priority for fiscal year (FY) 2005 (USFDA/CFSAN, 2004a) to determine if a revision to the policy was necessary in order to provide an adequate level of public health protection. While the final results of this scientific evaluation have not yet been released, comprehensive risk assessments and policy discussions occurring in other countries may have an impact on future U.S. regulatory policy.

International Perspectives

Internationally, countries often differ in their food inspection and certification systems as a result of differences in the prevalence of certain food safety hazards, differing views on food safety risk management and differences in the historical development of food control systems (CAC, 2003). With the globalization of the food supply countries must work together to provide an appropriate level of sanitary protection (ALOP) in order to facilitate fair trade while protecting public health (CAC, 2003). Importing countries must describe how their own sanitary measures achieve their ALOP, while the exporting country must objectively demonstrate that its measures can achieve that ALOP (CAC, 2003) either qualitatively, quantitatively or through a combination thereof (FSANZ, 2004).

The concept that the same level of food safety can be achieved through various hazard control measures and inspection and certification systems is referred to as the principle of equivalence (CAC, 1997). Equivalence is determined when different safety measures applied to a specified food, achieve the same level of food safety as that

achieved by standard or traditional methods (FSANZ, 2004). Equivalence can also be applied to specific requirements in regard to premises and equipment, processes such as Hazard Analysis Critical Control Point (HACCP) programs and end product microbiological limits (FSANZ, 2004).

This principle of equivalence is currently recognized in the World Trade Organization (WTO) Agreements on the Application of Sanitary and Phytosanitary Measures (WTO, 1994a) as well as the Agreement on Technical Barriers to Trade (WTO, 1994b) which encourage member countries to employ safety measures that conform to international standards unless they are deemed insufficient to achieve a country's ALOP. Similarly member countries are encouraged to accept as equivalent, measures and regulations of other members, provided they are satisfied these alternative measures and regulations meet their ALOP (FSANZ, 2004).

This principle of equivalence has been applied most recently to the importation of raw milk cheese in Australia and New Zealand. According to the Australia New Zealand Food Standards Code (FSC; ANZFA, 2008a), milk for cheese manufacture is required to be heat treated by pasteurization or subjected to a minimum heat treatment of 62°C for no less than 15 seconds (thermization) followed by a 90 day holding period. Although not in accordance with this domestic policy, Australia had previously allowed the importation of raw milk hard and semi-hard cheeses from Switzerland for over 30 years. The importation of Swiss cheese was suspended in 1997 following a review of quarantine requirements by the Australian Quarantine and Inspection Service (AQIS) when it was realized that these cheeses were made from raw milk and therefore not in compliance

with the FSC (ANZFA, 1998). Although the National Food Authority advised the AQIS that certain cheeses made in Switzerland, under specific cheesemaking methods, achieved a level of public health and safety equivalent to that of pasteurization back in 1994, a formal application was never submitted and thus the FSC was never changed (ANZFA, 1998).

In response, the Swiss Federal Veterinary Office (SFVO) submitted a request to restore market access for Swiss hard and semi-hard cheeses made from unpasteurized milk. In this application the risk of foodborne illness due to *E. coli*, *Salmonella* spp., *Staphylococcus aureus*, *L. monocytogenes* and *Campylobacter jejuni/coli* in Swiss hard and semi-hard cheese was assessed. The microbiological risk assessment of the manufacturing protocols set out in the Swiss Federal Government Ordinances determined that the heating temperatures of milk, continual heating of the curd, the rapid acidification by the starter cultures as well as the intense brining and long ripening period (90 - 360 days) inactivates pathogenic microorganisms (ANZFA, 1998). As a result, and based on the knowledge that the SFVO oversees the management of these protocols, the importation of the Swiss Emmentaler, Swiss Sbrinz and Swiss Gruyère was reinstated (ANZFA, 1998).

The importation and sale of very hard cooked-curd cheeses (hard grating cheeses) manufactured in Italy from raw milk such as Grana Padano and Parmigiano Reggiano was originally permitted based on compliance with Volume 1 of the FSC which stated that adequate heat treatment of the milk could be tested in terms of alkaline phosphatase (AP) destruction. However, at the close of 2002, Volume 1 of the FSC was replaced by

Volume 2, which did not include AP activity as an adequate measure of pasteurization efficacy placing these cheeses out of compliance. Therefore, a similar scientific evaluation of the safety of Italian hard grating cheeses with regard to the pathogens of concern as outlined in the Swiss application was conducted. The evaluation concluded that the manufacturing processes for Grana Padano, Parmigiano Reggiano, Romano, Asiago and Montasio achieved a 5 log reduction in bacterial pathogen levels, which is currently used as a benchmark for obtaining microbiologically safe products. The low moisture content (<36%), curd cooking temperatures and extensive aging was shown result in the inactivation of milkborne pathogens, if present. In 2002, Volume 2 of the FSC was amended to permit the sale of very hard cheeses (<36% moisture) manufactured from raw milk provided they are aged for a minimum of 6 months at a temperature of at least 10°C (ANZFA, 2002).

In 2004, France submitted an application to amend the FSC once again to allow for the sale of Roquefort cheese, a traditional French blue cheese made from raw sheep's milk. Roquefort maintains Appellation d'origine contrôlée (AOC) status and can therefore only be produced in a specific region of France. As with previous applications, a scientific evaluation of product safety regarding pathogens of concern was conducted, with the addition of *Brucella melitensis* and *Coxiella burnetii*. In contrast to the hard and very hard cheese varieties of previous applications, pathogen control for Roquefort is rooted in the implementation of an effective HACCP plan. These strategies rely heavily on microbiological testing (including final product) and the use of standard operating procedures (SOPs) and good manufacturing practices (GMPs) controlled by the

Confederation of Roquefort Producers (CRP). Although the manufacturing process for Roquefort is not fully bactericidal, the rapid acidification of milk and/or curd (decrease in pH from 6.5 to <5.0 within 6-8 hours and then to pH 4.8 within 24 hours), the reduction in water activity (~ 0.92) and the 90 day aging period aid in reducing potential hazards and therefore must be monitored (ANZFA, 2005).

The final step prior to approval of this application involved an onsite verification audit to verify the integrity of the regulatory control implemented by the CRP as enforced by the French Government including the inspection of facilities along the entire supply chain, examination of HACCP plans and documentation as well as the results of routine monitoring and testing. The sheep dairy farms supplying milk to Roquefort producers, operating under the supervision of the Departmental Veterinary Services Directorates and the CRP while in compliance with the standards outlined in the Roquefort Decree, keep extensive records on animal health including veterinary treatments, breeding activity, and transport to and from the farm allowing for the traceability of final product back to the raw milk supply (ANZFA, 2005).

Based on the information provided by the French Government, various challenge studies, a review by Food Science Australia and a review of the scientific literature, FSANZ concluded that any pathogens, if present, "would be unlikely to survive or proliferate during the manufacture of Roquefort cheese..." and that "...the consumption of Roquefort cheese poses a low risk to public health and safety". This conclusion was supported by the finding that there have been no reported foodborne outbreaks attributed to the consumption of Roquefort (ANZFA, 2005). Although the changes in processing

requirements for cheese and cheese products outlined in Standard 1.6.2 of the FSC did not originally apply to New Zealand, as of August 2007 New Zealand opened its borders to the direct importation of the aforementioned raw milk cheeses from countries other than Australia (NZFSA, 2007).

As in the U.S., raw milk cheese policy in Canada is also facing possible amendments. Similar to the standards of the PMO, the Food and Drug Regulations of Canada (PHAC, 2004) permit the sale of raw milk cheese provided the cheese has been stored at a temperature of at least 2°C for a minimum 60 days. In 1996, Health Canada (HC) proposed that all cheeses offered for sale be made from pasteurized milk or meet specific conditions considered equivalent to pasteurization (HC, 1996a). This proposal was met with great opposition from cheesemakers, notably in the province of Quebec. Following extensive consultations, HC withdrew this proposed amendment based on information provided by the Scientific Expert Advisory Committee that HC created to review the submissions made by industry, consumers and stakeholders during the consultation period (HC, 1996b). In response to the findings, the committee agreed that raw milk soft and semi-soft cheeses pose a higher risk than that of hard cheeses made from raw milk.

Recent cheese related outbreaks in Canada have been linked to an array of sources and products. One notable case involving a semi-hard cheese occurred in 2003 where 13 cases of *E. coli* O157:H7 infection, resulting in two deaths, were linked to the consumption of raw milk Gouda-style cheese in Alberta. Investigations revealed that the outbreak strain survived in the implicated cheese up to 104 days (Honish et al. 2005).

The first outbreak of listeriosis in Quebec, sickening 17 people, was attributed to the consumption of heat treated soft and semi-hard cheese. While raw milk from the cheese vat tested negative, 56 packages representing four different cheese varieties obtained from the maturing room tested positive for *L. monocytogenes*. Contamination from soil during renovations was cited as the likely source of contamination (Gaulin et al. 2003). Two additional outbreaks of listeriosis occurred in British Columbia in 2002. The first, linked to the consumption of pasteurized goat's and cow's milk cheese contaminated with *L. monocytogenes* from cheese ripening solutions (presumably cheese surface washing solutions), resulted in 48 cases of illness and three deaths (Wilcott, 2007). In the second outbreak *L. monocytogenes* isolated from a water cistern is believed to have contaminated pasteurized milk soft cheese resulting in 82 cases of illness (Wilcott, 2007).

In response to these outbreaks, acknowledging that *L. monocytogenes* represents a serious risk post-processing, Health Canada sought to develop a policy to replace the 60 day aging requirement for soft and semi-soft cheeses. It was recognized that the extended storage of these cheese varieties may allow for the growth of foodborne pathogens such as *L. monocytogenes* and may result in a spoiled and unpalatable product. The new policy would focus on strengthening production criteria and end-product testing coupled with stringent raw milk production when the milk is intended for the manufacture of raw milk soft and semi-soft cheeses. The implementation of a new Code of Practice would emphasize improved process controls and strengthen end product testing to achieve safety equivalent to that provided by pasteurized products (Farber, personal communication). In Europe, AOC soft cheeses such as the surface-ripened

Camembert de Normandie, are required to be manufactured from raw milk in order to receive AOC status where safety is not attained through aging (although aged a minimum 21 days), but through regulations specified in European Union (EU) Directives (N°2073/2005) which establish microbiological criteria dependent upon whether cheese is made from raw versus thermized milk.

The general hygiene requirements for all food business operators in the EU are laid down in Regulation N°852/2004 (EU, 2004a) and more specifically for foods of animal origin in Regulation N°853/2004 (EU, 2004b). This “farm to fork” or “stable to table” approach to food safety includes primary production (farmers) in food hygiene legislation. One key component is the requirement for food business operators (except farmers) to implement and maintain permanent procedures, based on HACCP principles in addition to good hygiene practices. Directive N°853/2004 outlines health requirements for raw milk production and hygiene on milk production holdings not much unlike those detailed in the PMO. The directive also includes microbiological criteria for raw milk used to prepare dairy products measured as a rolling geometric average over a two-month period, with at least two samples per month to account for seasonal variation. Somatic cells are similarly measured over a three-month period, with at least one sample per month, unless the competent authority specifies another methodology to take account of seasonal variations in production levels. Although the limits are similar to those outlined in the PMO for pre-pasteurized milk, a distinction is made between raw milk for heat treatment and that for the production of raw milk products. In general, as with the PMO, no standards are provided for the presence of pathogenic bacteria with the exception of

Brucella and *Mycobacterium*. The PMO (USFDA, 2003) requires prepasteurized milk to be from herds “officially free” from tuberculosis and brucellosis as certified or determined by the USDA whereas this requirement only applies to the sale of raw milk direct to consumers in the EU. Additional microbiological criteria for foodstuffs are outlined in commission regulation N°2073/2005 (EU, 2005). Food business operators must assure that cheeses made from raw or heat treated milk on the market do not contain *Salmonella* spp. in any of five 25-gram samples as determined by the International Standards Organization (ISO) standard 6579. Testing for coagulase positive staphylococci varies depending on the extent of heat treatment applied to the milk used for manufacture. This value, based on a three class sampling plan ($n=5$, $c=2$, $m=10^4$, $M=10^5$), is similar to that of the Compliance Guidelines enforced in the U.S. with one major exception. In the case of raw (ISO standard 6888-2) and heat treated milk (ISO standard 16649-1 or 2) samples must be taken at the time during the manufacturing process when the number of staphylococci is expected to be the highest as opposed to when the cheese is already on the market. Moreover, stricter limits are placed on cheeses manufactured from heat treated and pasteurized milks.

Another major deviation in cheese regulatory policy between the U.S. and the EU is related to allowable limits of *L. monocytogenes*. While the U.S. maintains a “zero tolerance” policy in all foods with the presence of the organism deeming the food adulterated (USFDA, 2005), the EU differentiates between those that can and cannot support the growth of this pathogen (other than those intended for infants and for special medical purposes). For RTE foods that support *Listeria* growth the food must not contain

any viable cells in any of five 25-gram samples before the food has left the immediate control of the food business operator who produced it (ISO standard 11290-1 and 2). This same product may contain up to 100 colony forming units (CFU) per gram by the end of its shelf life as is the case for RTE foods unable to support the growth of *L. monocytogenes*. Food business operators manufacturing RTE foods, which may pose a *L. monocytogenes* risk for public health, are also required to sample the processing areas and equipment for *L. monocytogenes* as part of their sampling scheme for HACCP validation (ISO standard 18593).

These recent changes in regulatory practice in other industrialized nations may soon have an influence on U.S. policy. For example in 2003 the FDA in association with the USDA/FSIS released a Quantitative Assessment of Relative Risk to Public Health from Foodborne *Listeria monocytogenes* Among Selected Categories of Ready-to-Eat Foods (USFDA/USDA/CDC, 2003). This assessment examined the available scientific data to predict the relative risks of illness and death, among various age groups, from the consumption of certain RTE foods. With the heterogeneity of virulence observed among different isolates under consideration, dose-response curves suggest that the relationship between pathogen levels ingested and the incidence of listeriosis greatly depends on the immune status of the individual where lower levels of contamination would most likely effect immunocompromised hosts. These models also suggest that exposure to low doses of *L. monocytogenes* in the general population presents a lower relative risk of contracting listeriosis than previously thought. This is an important consideration as most RTE foods contain either non-detectable or low levels of *L. monocytogenes* that

consumers in the U.S. are exposed to on a regular basis without apparent harm. Levels ≤ 100 CFU/g at the time of consumption were shown to pose a low level of risk to consumers while the low proportion of highly contaminated foods happens to cause a disproportionately high percentage of listeriosis cases (USFDA, 2003). Similarly the risk characterization of *L. monocytogenes* in ready-to eat foods conducted by FAO /WHO released in 2004 (FAO /WHO, 2004) found that nearly all cases of listeriosis are associated with the consumption of foods that did not meet current standards for levels of *L. monocytogenes* in foods, whether the standard is “zero tolerance” or 100 CFU/g. It was further stated that preventing the occurrence of high levels of contamination at consumption would have the greatest impact on reducing illness rates (FAO /WHO, 2004). Currently domestic policies are undergoing reexamination to determine whether or not public health could be enhanced by using a standard such as <100 CFU/g at the time of consumption in order to focus regulatory attention and compliance on the small percentage of servings containing high levels of contamination (USFDA, 2003; USFDA, 2004). Establishing critical limits is also warranted economically as recalls of products due to the zero tolerance policy can be financially devastating for manufacturers (Wulff et al. 2006).

Pathogens of Concern

Based on regulatory policies, compliance guidelines and risk assessments such as those previously described, in addition to the results of epidemiologic and pathogen challenge studies yet to be discussed, the pertinent foodborne pathogens of public health significance currently associated with raw and pasteurized milk cheese include *S. aureus*,

Shiga-toxin producing *E. coli* (notably O157:H7), *Salmonella* spp., and *L. monocytogenes*.

Staphylococcus aureus

Staphylococcus aureus

S. aureus is a nonsporeforming, facultatively anaerobic Gram-positive coccus-shaped bacterium that is both catalase and coagulase positive. Cells are typically arranged singly, paired, or in grape-like clusters (Le Loir et al. 2003). Staphylococcal food poisoning occurs not as the result of the ingestion of the organism itself, but through ingestion of one or more of the 14 staphylococcal enterotoxins (SE A-N) produced by some strains of *S. aureus* (Le Loir et al. 2003). With 30-50% of the population carrying *S. aureus* in their nostrils and on skin and hair, foods can become contaminated quite readily prior to or following heat treatment during processing and handling (Le Loir et al. 2003).

S. aureus has been shown to grow in foods over a wide range of water activity (a_w) levels. Water activity is a measure of the energy status of water in a food defined by the the vapor pressure of the water in a sample divided by that of pure distilled water at that same temperature. Generally a_w is described as a measure of the amount of “bound” water or water unavailable for microbial use. While growth limits vary in foods, *S. aureus* has been shown to grow in laboratory media at a_w levels as low as 0.86 (Genigeorgis, 1989). Enterotoxin production under various a_w levels differs by toxin type with SEA (Qi and Miller, 2000) and SED (Ewald and Notermans, 1988) produced over the range of a_w suitable for *S. aureus* growth, while SEB and SEC are more sensitive to

changes in a_w (Ewald and Notermans, 1988; Qi and Miller, 2000). *S. aureus* is also quite acid tolerant where some strains can grow in acidic environments as low as pH 4.0 (Smith et al. 1983). While SE production has been observed at pH values as low as pH 4.8 in buffered Brain Heart Infusion broth (BHI), most strains produce SE around pH 5.1 (Barber and Deibel, 1972). Once formed, SEs are resistant to the heat treatment and low pH conditions that destroy the bacteria that produce them (Le Loir et al. 2003).

Nature of disease

Staphylococcal food poisoning, also known as staphyloenterotoxigenosis or staphyloenterotoxemia, is the name of the condition caused by the ingestion of SEs. The onset of symptoms including nausea, vomiting, retching, diarrhea, and abdominal cramps, normally develops within 1 to 6 hours following ingestion of the contaminated food (Ryser, 2001). Depending on individual susceptibility to the toxin, the amount of toxin ingested and the general health of the affected individual, symptoms may also include headaches, cold sweats, rapid pulse, transient changes in blood pressure, prostration and dehydration. Recovery generally takes one to two days rarely resulting in complications or hospitalization (Ryser, 2001; FDA/CFSAN, 2008).

Outbreaks linked to milk and milk products

Outbreaks of staphylococcal poisoning have been linked to milk and milk products for over 100 years with *S. aureus* emerging as a major milkborne pathogen of concern by the 1930s (Ryser, 2001). The proportion of dairy related illnesses from staphylococcal poisoning in the U.S. has, however, decreased substantially in the past 40 years as a result of increased monitoring of mastitis in dairy cattle coupled with improved

sanitation and the implementation of pasteurization (Ryser, 2001). Despite similar improvements *S. aureus* is the leading cause of foodborne disease related to milk and milk products in France (De Buyser et al. 2001) possibly resulting from the use of raw milk. Most outbreaks linked to the use of raw milk have been traced to mastitic dairy cows whereas processed products are typically contaminated post-pasteurization through improper handling and human transmission (Ryser, 2001).

Although readily inactivated by standard heat treatments, foodborne outbreaks related to pasteurized products occur as the result of SE production prior to heat treatment or as a result of post-pasteurization contamination. Notable outbreaks occurring in the U.S. include 16 cases of staphylococcal food poisoning in 1981 linked to the consumption of pasteurized milk cheese (Altekruse et al. 1998). In this case milk was contaminated post-pasteurization prior to the addition of starter culture allowing for *S. aureus* to grow, resulting in SE production (Le Loir et al. 2003). In 1985, 860 illnesses were linked to the consumption of pasteurized milk. Prior to pasteurization the raw milk, contaminated with *S. aureus*, was improperly stored allowing for pathogen growth and subsequent toxin production (Evenson et al. 1988). Although the milk was later pasteurized thus inactivating viable *S. aureus*, the heat had little effect on the preformed toxin (Le Loir et al. 2003).

Notable outbreaks related to cheese are relatively rare as *S. aureus* is generally considered a poor competitor in the presence of active starter culture. An outbreak of staphylococcal poisoning sickening 42 persons was linked to Cheddar, Monterey and Kuminost cheeses manufactured from pasteurized milk contaminated post-pasteurization.

Delayed starter activity and abnormal acid development likely contributed to pathogen growth and subsequent toxin production (Ryser, 2001; Zehren and Zehren 1968a, 1968b). Contaminated raw milk has also been implicated in cheeseborne infection including 28 cases of illness from December 1984 to January 1985 traced to raw sheep's milk cheese in Scotland where SEA was present in the incriminated cheese in the absence of culturable organisms. Contamination was associated with post-infection carriage and clinical illness in sheep on the farm. Follow up investigations revealed that *S. aureus* isolates capable of producing SEA were sporadically present in the bulk tank milk for nearly two years in the absence of clinical illness (Bone et al. 1989). Although the details are unclear, Minas-type cheese heavily contaminated (7-8 log CFU/g) with *S. aureus* strains capable of producing enterotoxins A, B, D, and E (Sabioni et al. 1988) was implicated in outbreaks in Brazil in 1987 (Sabioni et al. 1988) and 1993 (Pereira et al. 1996).

Incidence in milk

As a major causative agent of mastitis, *S. aureus* is one of the most common contagious pathogens infecting dairy cows, and is commonly isolated from raw cow (25.1%; De Reu et al. 2004), goat (31.7%-38%; Tham et al. 1990), and sheep's milk (33.3%; Tham et al. 1990). Some surveys report prevalence rates as high as 75% and 96.2% for cow and goat milks, respectively (Jorgensen et al. 2005).

Behavior and enterotoxin production in cheese

The behavior and enterotoxin production of *S. aureus* in cheese varies depending on cheese type, activity and type of starter culture utilized (Meyrand et al. 1998) as well

as the presence of acidic byproducts, decreasing pH, and competition for nutrients (Genigeorgis, 1989). Without the addition of starter bacteria, *S. aureus* naturally occurring in raw milk (at low initial levels) may exhibit growth during manufacture of rennet coagulated fresh soft cheese (i.e. Tenerife), carrying over into young cheese. *S. aureus* levels typically decline shortly thereafter in the presence of dense populations of other raw milk flora (Zarate et al. 1997). Growth appears to be limited in fresh soft cheeses prepared by lactic coagulation from the activity of starter culture (Hamama et al. 2002).

Assuring low initial levels of *S. aureus* in milk (raw or pasteurized, ≤ 3 log CFU/ml) may also prevent the formation of toxin (SEC; Hamama et al. 2002). Vernozy-Rozand and colleagues (1998) observed the marked decline in *S. aureus* counts during the draining, salting and ripening of goats' milk lactic cheese. Negligible levels of SEA and a decline in *S. aureus* population levels were detected from initial milk populations of 5-6 log CFU/ml, with population declines attributed to low pH and the presence of salt. *S. aureus* has been shown to survive better during the manufacture and ripening of Camembert-type cheese manufactured from goat's milk where it is likely that the increase in pH provides a favorable environment for the organism. While SEA could be detected in cheeses when counts exceeded 6 log CFU/g of cheese, SEA was undetectable in most cheeses when initial counts in milk were ≤ 3 log CFU/ml (Meyrand et al. 1998).

S. aureus has also been shown to survive the manufacture and ripening of hard cheeses such as Manchego (Gomez-Lucia et al. 1992) resulting in SEA and SED production in cheeses made from milk with initial counts > 4 log CFU/ml. This

observation was attributed to *S. aureus* multiplication prior to pH reaching inhibitory levels as the result of active starter culture. Extensive heating such as that employed in the manufacture of processed cheese (80-85°C) readily inactivates *S. aureus* (Glass et al. 1998). Furthermore, *S. aureus* is unable to grow when introduced as a post-processing contaminant on the surface of processed cheese and therefore unable to produce SE (Glass et al. 1998).

In general, raw milk contaminated with low levels (240 CFU/ml; Jorgensen et al. 2005) of *S. aureus* does not necessarily present a food safety risk as virulence lies in the production of heat stable enterotoxins, which generally occurs when populations exceed 5 log CFU/ml. As a result of the variation in survival and enterotoxin production by *S. aureus* in cheese, the European Union (EU) requires that analyses for *S. aureus* be performed at the time during cheesemaking when the populations are expected to be the highest as opposed to counts at the time of release for sale at retail (EC, 2004). If values greater than 100,000 CFU/ml are detected, the cheese batch must then be tested for the presence of enterotoxins (EC, 2005).

Overall, initial milk population levels appear to have the strongest influence on levels attained in cheese (Delbes et al. 2006) while subsequent growth is influenced by starter activity and acidification. Therefore, the best protection from enterotoxin production in cheese of all varieties begins with assuring populations of *S. aureus* in milk, both raw and pasteurized, is kept low (under 100 CFU/ml; Delbes et al. 2006). Active starter cultures and rapid acidification will inhibit the growth and thus help control enterotoxin production thereafter.

Salmonella spp.

Genus *Salmonella*

Salmonellae are facultatively anaerobic, straight, usually motile (by peritrichous flagella) Gram-negative rods (0.7-1.5 x 2-5µm) belonging to the family *Enterobacteriaceae* (Holt et al. 1994). According to the most recently published classification guidelines, the genus *Salmonella* contains two species *S. enterica* (synonym: *S. choleraesuis*) and *S. bongori* (synonyms: *S. enterica* subsp. *bongori*, and *S. choleraesuis* subsp. *bongori*). *S. enterica* is further subdivided into six subspecies (synonyms): *S. enterica* subsp. *arizonae* (*S. arizonae*, and *S. choleraesuis* subsp. *arizonae*), *S. enterica* subsp. *diarizonae* (*S. choleraesuis* subsp. *diarizonae*), *S. enterica* subsp. *enterica* (*S. choleraesuis* subsp. *choeraesuis*, *S. enteritidis*, *S. paratyphi*, *S. typhi*, and *S. typhimurium*), *S. enterica* subsp. *houtenae* (*S. choleraesuis* subsp. *houtenae*), *S. enterica* subsp. *indica* (*S. choleraesuis* subsp. *indica*), and *S. enterica* subsp. *salamae* (*S. choleraesuis* subsp. *salamae*; Tindall et al. 2005). In 2005, a new species *S. subterrannea* was validly published (Anonymous, 2005). Salmonellae are further subdivided in the Kauffmann-White's scheme into over 2,500 serotypes based on the characterization of the somatic (O), flagellar (H), and capsular (Vi) antigens (Holt et al. 1994). The greatest number of serotypes belong to *S. enterica* ssp. *enterica*, typically named for the geographic location of an outbreak (i.e. Montevideo, referred to as *S. Montevideo*), or by their antigenic formula.

Optimum growth for salmonellae occurs at 35 to 37°C, while minimum temperatures allowing for growth range from 5.5 to 6.5°C depending on serotype. In

general, foods held below 5°C do not support the growth of salmonellae. Although optimal growth pH is between 6.5 and 7.5, salmonellae can grow readily in more acidic environments. The minimal pH in which growth is observed varies depending on acid type, temperature, available oxygen, growth medium, level of inoculum and serotype (El-Gazzar and Marth, 1992).

Nature of disease

Despite the vast number of serotypes, all salmonellae are considered pathogenic, producing infections from mild gastroenteritis to typhoid fever (Ryser, 2001).

Salmonellosis generally a self-limiting gastroenteritis similar to intestinal influenza, and is believed to be grossly underreported. Onset of non-typhoidal salmonellosis typically occurs 12 to 36 hours after ingestion of the contaminated food characterized by nausea and vomiting; symptoms which tend to subside within a few hours (Ryser, 2001). The sudden onset of nausea, vomiting, chills, fever and abdominal pain, resembling acute appendicitis, is typically followed by diarrhea (El-Gazzar and Marth, 1992; Ryser, 2001). In some cases septicemia can occur as a complication of gastroenteritis which can be fatal in immunocompromised hosts. Prolonged septicemic infections can result in localized tissue and organ infections, especially in those previously damaged or diseased (Ryser, 2001). The severity and duration of symptoms depends upon the type and amount of organisms consumed, as well as host susceptibility with illness typically lasting between 2 and 6 days (El-Gazzar and Marth, 1992).

One biochemically distinct serovar, *S. Typhi* is responsible for bacteremia-related enteric fever referred to as typhoid fever. Onset typically occurs within 8 to 15 days, and

sometimes as long as 30 to 35 days. Fever, usually accompanied by various other non-specific symptoms, peaks at 104-105°F within three to four days of onset and can last for weeks resulting in numerous complications and even death (Ryser, 2001). The high mortality rate of *S. Typhi* (10%; Walderhaug, 2003) compared to other *Salmonella* spp. can be reduced with the prompt administration of antibiotics. Following treatment most patients continue to shed *S. Typhi* in their feces for up to three months with some individuals actively shedding the organism for longer than a year perpetuating the infectious cycle (Ryser, 2001). In addition, long term sequelae such as arthritic symptoms can arise three to four weeks following initial infection in approximately 2% of cases (Walderhaug, 2003).

In 2005, *S. Typhimurium* was the most commonly implicated serotype in the U.S. accounting for 20% of *Salmonella* infections (CDC, 2007b). While multiple drug resistant (MDR) types of *S. Typhimurium* definitive type (DT) 104 were initially identified in the early 1980's in gulls and wild birds, nearly 70% of the 684 isolates recovered in the National Antimicrobial Resistance Monitoring System (NARMS) in 2004, were resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracyclines (ACSSuT type; Threlfall, 2000; CDC, 2007a). Moreover, most strains possess additional, chromosomally encoded resistance to trimethoprim and ciprofloxacin (Threlfall, 2000). The emergence of MDR types of salmonellae has become a major concern as infections with MDR *S. Typhimurium* DT104 result in greater morbidity and mortality when compared with non-resistant salmonellae (Helms et al. 2002). Recently, multi-drug resistant strains of *S. Newport*, the third most common serotype, have

emerged exhibiting resistance to amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (MDRAmpC type) as well as other third generation cephalosporins or fluoroquinolones (Austin et al. 2008; CDC, 2007a).

Outbreaks related to milk and milk products

Non-typhoidal salmonellae infect an estimated 1,412,498 people in the U.S. annually resulting in approximately 582 deaths. Ninety-five percent of these illnesses are believed to be foodborne. Additionally, an estimated 824 cases of foodborne illnesses are attributed to *S. Typhi*, most of which occur abroad (70%; Mead et al. 1999). As the first serovar to plague the U.S. dairy industry, *S. Typhi* was responsible for 50 to 80% of reported foodborne illnesses related to milk and milk products (primarily raw) until the 1940's (Bryan, 1983; Ryser, 2001). According to Ryser (2001) the results of challenge studies conducted in the first half of the 20th century indicating that *S. Typhi* could persist in Cheddar cheese led to the present regulations requiring Cheddar, and other cheeses, made from raw milk to be aged at least 60 days at a temperature no less than 1.7°C (35°F). With the implementation of routine pasteurization and improved sanitation standards after World War II, the proportion of milkborne cases of typhoid fever dropped significantly with no dairy related cases reported in the U.S. since the 1950s (Bryan, 1983; Ryser, 2001).

Numerous documented outbreaks of non-typhoidal salmonellosis have been linked to the consumption of both raw and pasteurized milk and milk products since as early as 1965 (Ryser, 2001; Bryan, 1983). According to Headrick and colleagues (1998),

salmonellae were responsible for more than one quarter (331 cases) of the 46 raw fluid milk associated disease outbreaks reported in the U.S. from 1973 to 1992; second only *Campylobacter* spp. In 1984, 16 cases of *S. typhimurium* gastroenteritis were reported in persons associated with a convent in western Kentucky that bought Grade A raw milk and pasteurized it onsite with no time-temperature record or head space heater. Based on the evidence of inadequate pasteurization and the results of plasmid profiling of milk and human isolates it was concluded that inadequate milk pasteurization was responsible for the outbreak (Adams et al. 1984). In 1985, more than 16,000 culture-confirmed cases of *S. typhimurium* infection across six Midwest states were linked to the consumption of 2% pasteurized milk (Lecos, 1986). From 2002-2003 a multistate outbreak of *Salmonella* serotype *typhimurium* infections in Illinois, Indiana, Ohio, and Tennessee were associated with raw milk and milk products. While the source of contamination was not determined, it was suggested that contamination of milk may have occurred during the milking, bottling, or capping process as four barn workers who milked the cows, bottled the milk, and made ice cream had asymptomatic *S. typhimurium* infections (Holt et al. 2003).

Aside from milk, numerous outbreaks of salmonellosis involving cheese have been reported since 1976 (Ryser, 2001). That year, ongoing *Salmonella* surveillance in Colorado detected an outbreak of *S. Heidelberg* that eventually resulted in 339 cases of gastroenteritis linked to the consumption of pasteurized milk Cheddar cheese (Fontaine et al. 1980). Raw cheesemilk was held unrefrigerated, although insulated, for 1 to 3 days prior to pasteurization. It is unknown if any deficiencies in the pasteurization process occurred as no phosphatase samples were taken. It is likely that non-compliance with

good manufacturing practices coupled with inadequate control programs at the cheese plant were the causative factors. Levels of *S. Heidelberg* in contaminated cheeses were estimated to be <1 organism per 100g suggesting a low oral infectious dose (Ryser, 2001). Six years later an outbreak of *S. Muenster* in Canada was linked to Cheddar cheese produced from the raw milk of an infected herd with at least one mastitic cow shedding the outbreak organism (El-Gazzar and Marth, 1992; Ryser, 2001). In 1984 the single largest epidemic of salmonellosis in Canada with more than 2,000 confirmed cases reported in the four Atlantic Provinces and Ontario was linked to cheese manufactured from either pasteurized or heat treated (66.7° C for 16 sec) milk. *Salmonellae* were present in contaminated cheese at approximately <10 organisms per 100g, similar to the low levels observed in the 1976 outbreak previously mentioned (D'Aoust et al. 1985; Ratnam and March, 1986). Further investigation revealed that an employee manually overrode the pasteurization process shutting down the pasteurizer while milk continued to flow through and into the vat (Johnson et al. 1990). Molecular subtyping later revealed that the implicated cheese likely had two sources of contamination. One subgroup (I) among the *S. Typhimurium* phage type 10 isolates was obtained from raw milk and dairy cows while both group I and II plasmid patterns isolated from cheese occurred among isolates recovered from workers at the dairy as well as their family members. Plant inspections revealed these employees involved in either the production and or packaging of cheese used their bare hands to transfer cheese to a forming machine further implicating ill workers as a likely source of contamination (Bezanson et al. 1985).

In 1989, a multistate outbreak of the rare serotypes *S. Javiana* and *S. Oranienburg* was epidemiologically linked to consumption of contaminated Mozzarella cheese and shredded cheese products in Minnesota and Wisconsin. Case control studies traced the source of contamination to a single plant (plant X) as well as cheese that had been shredded at processing plants that also shredded cheese manufactured at plant X (Hedberg et al. 1992). With no deficiencies in pasteurization noted, finished product was likely contaminated through handling by an infected employee or environmental contamination. It is believed that contaminated Mozzarella cheese sent to four processing plants for shredding cross-contaminated other cheese products at those plants as inspections later revealed that equipment was not routinely cleaned and sanitized between shredding cheese from different manufacturers. The low levels of salmonellae in contaminated samples (0.36 MPN/10 g and 4.3 MPN/100g) provide further evidence for the low infectious dose of *Salmonella* spp. (Hedberg et al. 1992). That same year an outbreak *S. Dublin* infections linked to the consumption of an Irish soft unpasteurized cow's milk cheese occurred in England and Wales resulting in 42 cases of illness (Maguire et al. 1992).

A large nationwide outbreak of salmonellosis linked unpasteurized goats' milk cheese occurred in France over the course of three months in 1993. This outbreak involved 273 cases of *S. paratyphi* B infection resulting in one death (Rampling, 1996). While contaminated milk from one of the 40 suppliers was implicated, the specific source of infection (human, animal, or environmental) was not identified (Desenclos et al. 1996). Unpasteurized soft cheese product produced on a farm and sold at farmer's markets was

linked to 82 clinical cases of *S. Berta* infection in Ontario in 1994. Subtyping results indicated that patient, cheese, and chicken isolates were genetically indistinguishable suggesting that the cheese was cross-contaminated by the chicken carcasses during production (Ellis et al. 1998). Three years later consumption of Morbier, a raw milk soft cheese, was implicated in a community wide outbreak of *S. Typhimurium* infection in the Jura district of France (De Valk et al. 2000).

In 1998, a major Canadian outbreak of *S. Enteritidis* phage type 8 which sickened more than 800 people, most of whom were children, was associated with the consumption of contaminated pasteurized Cheddar cheese contained in pre-packaged lunch products (Ahmed et al. 2000; Ratnam et al. 1999). *S. Enteritidis* phage type 8 was again implicated in two community outbreaks in the southwest of France in 2001 linked to the consumption of Cantal cheese manufactured from raw milk (Haeghebaert et al. 2003). Sixteen cases of *S. Oranienburg* infection occurred in the Austrian province of Tyrol in 1999 that were traced to cow's milk cheese locally produced on an alpine farm. According to the tenant of the alpine pasture, chickens were allowed to roam free and had repeated access to the dairy. Follow up investigations yielded *S. Oranienburg* from both environmental and fecal samples taken from a chicken coop implicating the chickens as the source of the milk and cheese contamination (Allerberger et al. 2000).

In early 1997, health officials in Washington State noted a 5-fold increase in salmonellosis cases among the Hispanic population of Yakima County with 54 culture confirmed cases reported, a majority of which were ACSSuT type DT104 or DT104b. Implicated cheeses were manufactured by two individuals in separate towns with raw

milk from the same dairy. That same year, two overlapping outbreaks involving 110 confirmed cases of MDR *S. Typhimurium* DT104 and DT104b were associated with the consumption of fresh homemade Mexican-style cheese produced from raw cow's milk in California (Cody et al. 1999). In both cases cheeses were illegally manufactured from raw milk in the home and sold. Also in 1997, a small outbreak of MDR salmonellosis was linked to the consumption of raw milk and contact with infected animals was reported in Vermont (Marcus et al. 1997). More recently, between early 2006 and 2007, 85 culture-confirmed cases of *S. Newport*, were identified in northeastern Illinois. Genetically indistinguishable isolates of *S. Newport* were recovered from all 85 patients, an aged Mexican-style cheese (Cotija), and raw milk from a local dairy (Austin et al. 2008).

As previously outlined, outbreaks of cheeseborne salmonellosis are generally the result of a few common practices including the use of raw milk or inadequate milk pasteurization coupled with non-compliance with good manufacturing practices and inadequate control programs. Raw milk can be contaminated by an infected herd while pasteurized milk can be cross-contaminated by other farm animals such as chickens. Numerous outbreaks have also resulted from contamination by ill employees. These areas represent focal points in the control of salmonellae in both raw and pasteurized milk cheese.

Incidence in milk

Salmonella spp. can infect dairy cattle and other ruminant animals with symptomatic or asymptomatic fecal shedding (Ryser, 2001). As a result, the intestinal

tracts of dairy cows serve as a major reservoir for human foodborne salmonellosis via fecal contamination (El-Gazzar and Marth, 1992). Raw milk, even from healthy animals, occasionally contains *Salmonella* as some strains, although uncommon, have been associated with mastitis and are therefore shed in milk during collection (Fontaine et al. 1980). *Salmonella* spp. incidence rates reported for raw milk in Europe range from 0% to 2.9% (Rea et al. 1992, Desmaures et al. 1997; De Reu et al. 2004). While low incidence (0.17%) has been reported in Canada (Steele et al. 1997), higher isolation rates from 1.5% to 8.9% of cow's milk samples have been reported in the U.S. (McManus and Lanier, 1987; Murinda et al. 2002; Rohrbach et al. 1992; Jayarao and Henning, 2001; Jayarao et al. 2006; Van Kessel et al. 2004) depending on geographic location. Similarly, Hassan and colleagues (2000) isolated *Salmonella* spp. from 1.5% of milk filters from New York dairy herds, a significantly lower rate than observed for *L. monocytogenes*. Although incidence data for small ruminant milk is limited, detection of *Salmonella* spp. has been reported in goat's milk (Foschino et al. 2002).

Wells and colleagues (2001) recovered *Salmonella* spp. from 5.4% of fecal samples obtained from dairy cows representing 91 herds from 19 states. When comparing recovery rates by herd size it was noted that farms with less than 100 animals had a lower (0.6%) incidence rate than those with more than 100 (8.8%). This suggests that small-scale producers maintaining small herds would display a comparatively low incidence of *Salmonella* spp. shed in feces. Assuming the incidence pathogens in milk is lower than that of fecal samples; raw milk produced on small-scale farms would likely have a lower incidence of *Salmonella* spp. as well. It is important to consider that the

results of the aforementioned surveys were obtained using standard cultural procedures. In a more recent investigation the assay of milk samples by real-time PCR indicated that the prevalence of *S. enterica* in bulk tank milk may be substantially higher than previously reported (Karns et al. 2005).

Behavior in cheese

Early challenge studies demonstrated the survival of salmonellae in Cheddar cheese. In one such study, survival ranged from 2 to 9 months dependant upon pH as well as the type and amount of starter culture. Cheeses with abnormally high pH values as a result of starter culture failure displayed little inhibitory activity whereas cheeses with pH values between 5.2 and 5.3 resulted in inactivation (Hargrove et al. 1969). White and Custer (1976) examined the survival of *S. Newport*, *S. Newbrunswick*, and *S. Infantis* inoculated into cheesemilk during the manufacture and storage (4.5 or 10°C) of Cheddar cheese. They found that salmonellae, when initially present in large numbers in cheesemilk (~5 log CFU/ml), can survive as long as 9 months (El-Gazzar and Marth, 1992; Ryser, 2001). Similar results were obtained by Park and colleagues (1970) where salmonellae survived up to 7 and 10 months at 13°C and 7°C, respectively. It is likely that pathogen growth and survival was related to the relatively high cheese pH values (5.75 and 5.9). Moreover, the cheese in the latter study was of high moisture (~43%). Goepfert and others (1968) examined the fate of *S. typhimurium* during the manufacture and aging of stirred curd Cheddar cheese from cheesemilk inoculated with 1-3 log CFU/ml. Following an initial increase in cell numbers during manufacture, the number of salmonellae decreased by a factor of 10,000 after 10 to 12 weeks at 13°C, and 14 to 16

weeks at 7.5°C. It is important to note that the mean pH of the cheese after overnight pressing in this study was 5.1, far lower than in the aforementioned studies. Moisture and salt contents, however, were not provided.

While the fate of salmonellae has been studied in various cheeses, most have employed non-stressed cultures which do not account for the adaptive responses to acidity and osmolarity that may promote growth and survival in environments hostile to nonadapted cells (Leyer and Johnson, 1992). Acid-adapted cells of *S. Typhimurium* display increased resistance to inactivation by organic acids commonly present in cheese including lactic, propionic and acetic acids. Acid-adapted cells have also shown enhanced survival during milk fermentation by lactic acid bacteria when compared to nonadapted cells. Similarly acid-adaptation promotes persistence of *S. Typhimurium* as a surface contaminant in Cheddar, Swiss, and Mozzarella cheeses when held at 5°C (Leyer and Johnson, 1992). Cold adapted *S. Enteritidis* has also been shown to survive and grow during the storage of both low and full fat cream cheese (Smith-Palmer et al. 2001).

Escherichia coli

Escherichia coli

E. coli are facultatively anaerobic, Gram-negative rods of the family *Enterobacteriaceae* commonly found in soil and water. *E. coli* also constitute part of the normal intestinal flora of humans and other warm-blooded animals (Holt et al. 1994). While many strains are innocuous inhabitants, some are capable of causing disease in humans. Strains of *E. coli* are serotyped on the basis of three major surface antigens: O (somatic), H (flagellar), and K (capsular). Specific serotypes and serogroups are often

associated with certain clinical syndromes and therefore serve as identifiable chromosomal markers that correlate with specific virulence factors (Nataro and Kaper, 1998).

Pathotypes

Currently there are at least six well known classes of *E. coli* known to cause foodborne gastrointestinal disease in humans (Kaper, 2005) categorized by their virulence properties, pathogenic mechanisms, clinical syndromes and O:H serogroups (Nataro and Kaper, 1998). Enteropathogenic *E. coli* (EPEC) are typically associated with infant diarrhea in developing countries characterized by attaching and effacing (AE) lesions resulting from the intimate bacterial adherence to epithelial cells and degeneration and effacement of intestinal cell microvilli (Law et al. 2000). Enterotoxigenic *E. coli* (ETEC) are also associated with infant diarrhea in developing countries as well as travelers' diarrhea in individuals traveling to developing countries. In addition to a variety of intestinal colonization factors, ETEC strains produce heat stable (ST) and/or heat-labile (LT, similar to the cholera toxin) enterotoxins that stimulate the lining of the intestines resulting in excessive fluid secretion, thus producing diarrhea (Kaper, 2005). Enteroinvasive *E. coli* (EIEC) are so named for their ability to invade, proliferate in and subsequently spread between the epithelial cells of the colon, damaging the epithelial lining. This generally results in watery diarrhea often containing blood, mucus, and polymorphonuclear leukocytes typical of shigellae infections (Padhye and Doyle, 1992). Enteroaggregative *E. coli* (EAggEC) utilize fimbriae to bind to the cells of the small intestine in small clumps, hence their pathotype name. Subsequent production of a heat

stable enterotoxin and a cytotoxin are believed to cause diarrhea in young children (Padhye and Doyle, 1992). EAggEC are increasingly recognized as a cause of persistent diarrhea in adults in both developing and industrialized nations (Kaper, 2005). Similarly, diffusely adherent *E. coli* (DAggEC), initially defined based upon the diffuse pattern of adherence on cultured epithelial cells, have been implicated as a cause of human diarrhea (Kaper, 2005).

Shiga toxin-producing *E. coli* (STEC). STEC are so named for the presence of a specific virulence marker responsible for the production of verocytotoxins (toxic to Vero (green monkey kidney) cells in tissue culture) similar to the toxin produced by *Shigella dysenteriae*, and are thus often referred to as Shiga-like toxins Stx1 and Stx2. The genes encoding these toxins are carried on bacteriophages and could conceivably be acquired by *E. coli* of any serotype. Recent research has demonstrated that exposure to certain antibiotics not only results in increased toxin production, but also leads to mobilization of phage (Zhang et al. 2000). Although more than 200 serotypes of *E. coli* have been shown to produce shiga-like toxin, the majority of these are not thought to be pathogenic in the absence of other virulence factors. Therefore, the ability to produce toxin alone may be insufficient to cause disease (Law et al. 2000). The virulence of STEC is multifactorial, beginning with colonization of the bowel via the action of one or more adhesins followed by the release of toxins and other proteins which may assist with bacterial survival and multiplication. The production and release of toxins into the gut lumen causes intestinal damage followed by systemic sequelae (Law et al. 2000). The

variability in virulence between STEC strains has been observed among isolates obtained from unpasteurized dairy products (Fach et al. 2001).

Enterohemorrhagic *E. coli* (EHEC) are a subset of the broader STEC category displaying increased virulence with potentially high mortality (Law et al. 2000).

Following an incubation period ranging from 3 to 9 days, EHEC infections manifest as three principal illnesses: hemorrhagic colitis (HC), hemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP; Karmali, 1989). HC is characterized by the sudden onset of extremely painful abdominal cramps followed by watery diarrhea which later becomes grossly bloody (Padhye and Doyle, 1992). HUS is a disease triad of acquired hemolytic anemia, thrombocytopenia and renal failure (Padhye and Doyle, 1992) resulting from toxin-mediated damage to the kidneys. Endothelial damage triggers the clotting mechanism that may block capillaries of the kidneys and other organs leading to the buildup of waste products in blood (Padhye and Doyle, 1992) often requiring dialysis and blood transfusions. HUS is thought to complicate approximately 10% of EHEC infections with a mortality rate between 2-10% (Law, 2000), often resulting from the development of cardiovascular and central nervous system diseases such as heart failure, coma, seizures and hypertensive encephalitis (Padhye and Doyle, 1992).

Virulence. The ability of EHEC strains to cause disease is dependant upon the ability to adhere to the bowel mucosa and produce toxins, notably Stx2. The lack of fever presentation in infected patients suggests that *E. coli* O157:H7 is not invasive and does not enter the circulatory system. EHEC strains possess a locus of enterocyte

effacement (LEE) containing the genes necessary for AE including *eae*, which encodes for intimin, an outer membrane protein necessary for intimate attachment. Although other adherence factors have been identified, intimin has been the most studied adhesin. This LEE also contains the *tir* gene encoding the protein Tir, or translocated intimin receptor, which may also be necessary for cellular attachment. Most EHEC, including isolates of *E. coli* O157, solely produce the more cytotoxic Stx2 which can damage renal glomerular vasculature resulting in the development of HUS (Law et al. 2000). The increased pathogenicity of *E. coli* O157 strains may also be attributed to the comparatively higher production of Stx2 (Law et al. 2000). Since *E. coli* O157 is non-invasive, toxin mediated systemic damage is believed to result from toxin translocation from the gut lumen to underlying tissues. Toxin molecules, once bound to the target cell membrane can be internalized by receptor mediated endocytosis where, in some cells, they are degraded by lysosomes. However, in other cells the toxin may be processed in the golgi and endoplasmic reticulum resulting in a glycosidic subunit whose action inhibits peptide chain elongation in protein synthesis resulting in cell death (Law et al. 2000). STEC strains also contain a highly conserved *ehx* operon that encodes for the enterohemolysin, Ehx. Although the contribution to virulence is unclear, hemolysin production may be related to the lysis of erythrocytes present following toxin mediated damage to the bowel mucosa and underlying blood vessels. The release of hemoglobin from lysed erythrocytes may also serve as an iron source for O157 strains, thus stimulating growth and subsequent toxin production (Law et al. 2000). The

aforementioned virulence genes (*stx1*, *stx2*, *eae*, *ehx* etc.) also serve as markers for PCR assays.

It is believed that the ability of *E. coli* O157:H7 to induce an adaptive tolerance response (ATR) when exposed to mild acid conditions may confer a higher resistance to stronger acid conditions upon subsequent exposure (Jordan et al. 1999). Therefore, induction of an ATR following exposure to the mild acid conditions found in cheese may promote greater resistance to acid during passage through the stomach, thus reducing the infectious dose (Maher et al. 2001). While serotype O157:H7 is the most common and recognized serotype, non-motile variants (O157: NM) lacking a flagellar antigen, as well as serotypes O26:H11, O103:H2, O2:H5 and O111 are occasionally isolated from patients with hemorrhagic colitis or the hemolytic uremic syndrome (Su and Brandt 1995).

Outbreaks related to milk and milk products

There are four principal routes of EHEC infection including direct contact with infected animals, person to person transmission, transmission through the environment and foodborne transmission. First recognized as a foodborne pathogen in 1982 following two outbreaks of HC in Oregon and Michigan attributed to the consumption of undercooked beef patties (Riley et al. 1983), EHEC has emerged as a major pathogen of concern across almost all major food categories. In 1996, *E. coli* O157:H7 was added to the CDC's Foodborne Diseases Active Surveillance Network (FoodNet). Based on surveillance data, an estimated 62,000 foodborne cases occur annually in the U.S. resulting in approximately 1,800 hospitalizations and 52 deaths (Mead et al. 1999).

Foodborne outbreaks of *E. coli* O157:H7 have involved a wide range of food products. Those of bovine origin (Feng, 1995), including raw and pasteurized milk and milk products (Keene et al. 1997; Upton and Coia, 1994) and ground beef (including that from cull dairy cows; McDonough et al. 2000) are most commonly implicated. From 1982 to 2002, a total of 183 foodborne *E. coli* O157 outbreaks were reported in the U.S. While ground beef serves as the vehicle of infection most often identified, seven (4%) outbreaks were attributed to dairy products, four of which were linked to the consumption of unpasteurized fluid milk (Rangel et al. 2005). Similarly, Wachsmuth and colleagues (1997) reported that raw milk was responsible for 5% of O157:H7 outbreaks in the U.S. from 1982 to 1995. The only domestic outbreak linked to the consumption of cheese occurred in Wisconsin in 1998 where vats used to make raw milk Cheddar cheese were inadvertently used to make fresh cheese curds. The fresh curds, incorrectly labeled "pasteurized", were distributed and sold in six Wisconsin counties sickening 55 people (CDC, 2000). Around this time, cheese made with unpasteurized milk was the source of six cases of gastroenteritis in England (Strachan et al. 2005) including one case of HUS in a 12 year old boy (CDSC, 1998). Then in early 1999, three people from northeast England fell ill with *E. coli* O157 infections after consuming Cotherstone cheese made from unpasteurised cows' milk (CDSC, 1999a). Samples obtained from the dairy herd, slurry, and cheese production environment were all negative for *E. coli* O157 (CDSC, 1999b). That same year 60 cases had been confirmed in an outbreak of *E. coli* O157 infection in North Cumbria associated with the consumption of milk from a local farm resulting in 27 (11 adults and 16 children) hospitalizations, including three cases of HUS

(CDSC, 1999c,d) . Most recently in 2003, 13 cases of *E. coli* O157:H7 infection were linked to the consumption of Gouda cheese in Alberta, Canada resulting in 2 cases of HUS (Honish et al. 2005). In addition to cow's milk, raw goat's milk was recently implicated in an outbreak of *E. coli* O157:H7 affecting five people in British Columbia, Canada (PHAC, 2001). Although raw milk and milk products have been implicated in outbreaks, it appears as though contamination of milk may be uncommon (Coia et al. 2001).

Incidence in milk

Dairy cattle are a known reservoir of STEC, including O157:H7 (Wells et al. 1991). Early investigations revealed that *E. coli* O157:H7 can readily contaminate raw milk on the farm with contamination rates of 4.2 to 10% reported in the U.S. and 2% in Canada (D'Aoust, 1989; Padhye and Doyle, 1991). Studies have demonstrated the ability of *E. coli* O157:H7 to survive on pastures in soil and manure for extended periods of time (Bolton et al. 1999; Gagliardi and Karns, 2000; Lau and Ingham, 2001). Contaminated animal drinking water and birds may also serve as common vehicles for infection and a potential intervention site for on-farm control of dissemination (Shere et al. 1998; Wallace et al. 1997).

The presence of *E. coli* O157:H7 on a dairy farm, however, does not necessarily correlate to pathogen presence in raw milk as Wells and colleagues (1991) isolated O157:H7 in only 1 of 23 (4.3%) raw milk samples from a case farm in Wisconsin. Incidence also appears to be fairly low (0.75%) in the bulk tank milk of cull cows (Murinda et al. 2002b). Higher isolation rates including 3.8% in South Dakota and

western Minnesota (Jayarao and Henning, 2001) and 2.4% in Pennsylvania (Jayarao et al., 2006) have been reported for STEC. Although potentially pathogenic, none of the isolates in any of the aforementioned studies were of serotype O157:H7. Similarly, no O157:H7 were found in surveys conducted in southeast Scotland (Coia et al. 2001), Italy (Massa et al. 1999) or The Netherlands (Heuvelink et al. 1998) involving 500, 100 and 1011 raw milk samples, respectively.

Surveys examining the prevalence of *E. coli* O157:H7 in small ruminant milk are scant. Foschino and others (2002) reported an isolation rate of 1.7% for raw goat's milk in Italy. When present, population levels were low at 1.5 CFU/ml. Shiga toxin producing *E. coli* have also been detected in both goat and sheep milk at 16.3% and 12.7%, respectively (Muehlherr et. al. 2003). Although rare, milk and milk products can become contaminated as a result of post-processing contamination (Heuvelink et al. 1998; Morgan et al. 1993; Upton and Coia, 1994). However, it has been suggested that this serotype may be unable to survive in dairy processing environments and may be readily eliminated by sanitation practices and is therefore not widely disseminated (Ansary and Kaspar, 1997).

Behavior in cheese

Although the exact infectious dose for *E. coli* O157:H7 is unknown, it is thought to be as low as <100 organisms (Strachan et al. 2005). Challenge studies have shown that *E. coli* O157:H7 can grow at temperatures as low as 7°C in food products such as milk (Heuvelink et al. 1998) and likely survives during refrigerated storage in highly acidic environments as found in yogurt (Hudson et al. 1997; Massa et al. 1997), sour

cream, buttermilk (Dineen et al. 1998), apple cider (Miller and Kaspar, 1994) and cheese among others. An alternate sigma factor, *rpoS*, regulates genes required for acid tolerance allowing *E. coli* O157:H7 to survive at pH values as low as 2.5 for over 2 hours (Law et al. 2000). The *rpoS*-regulated proteins also cross protect *E. coli* O157:H7 against subsequent heat and salt challenges. *E. coli* O157:H7 has also been shown to survive the processing conditions of Feta (Hudson et al. 1997; Ramsaran et al. 1998), Colby, Romano (Hudson et al. 1997), Camembert (Ramsaran et al. 1998), smear rind (Maher et al. 2001) and Cheddar cheeses (Reitsma and Henning, 1996; Schlessner et al. 2006). Subsequent reduction in populations observed during the aging (<30 days) of Colby and Romano are likely the result of prolonged exposure to pH, presence of starter culture, temperature, and salt concentration (Hudson et al. 1997). Population inactivation during aging of smear rind cheese, whose pH increases following surface growth, is thought to result from the activity of antimicrobial substances produced by the surface flora (Maher et al. 2001). While *E. coli* O157:H7 may survive the initial steps in manufacture, exposure to elevated temperatures (80°C) during the stretching of Mozzarella cheese (Spano et al. 2003) and the cooking of cottage cheese (Arocha et al. 1992) results in complete pathogen inactivation. Studies have also shown that heating raw milk to 65°C for 17.6 seconds is sufficient for the destruction of *E. coli* O157:H7 (D'Aoust et al. 1988). In addition to these findings, Bowen and Henning (1994) failed to recover *E. coli* O157:H7 in 50 retail samples of natural cheese. Moreover, no *E. coli* O157 was detected in 153 soft and semi-soft cheeses made with raw cow, ewe and goat milk in a survey conducted in Belgium (Vivegnis, 1999).

Thus far this review has briefly outlined three organisms of public health concern associated with milk and milk products. As *L. monocytogenes* was the focus of this dissertation, it will be discussed in greater detail including overviews of environmental incidence and its role as a post-processing environmental contaminant as well as current methodologies for isolation and detection including subtyping techniques.

Listeria monocytogenes

Genus *Listeria*

Listeriae are short (0.5µ x 0.5-2µ), Gram positive, non-sporeforming, catalase and oxidase positive, facultatively anaerobic bacterial rods with rounded ends appearing almost coccoid at times. Cells most often occur singly or in short chains but may form long filaments. These bacteria are motile by peritrichous flagella at 20 to 25°C but not at the optimum growth temperature of 30 to 37°C (Holt et al. 1994). There are currently six recognized species of listeriae including *L. welshimeri*, *L. murrayi*, *L. grayi*, *L. innocua*, *L. ivanovii* and *L. monocytogenes*. Although there have been isolated cases of *L. ivanovii* causing disease in humans (Cummins et al. 1994), *L. ivanovii* is typically pathogenic to non-human mammals. *L. monocytogenes* is pathogenic to both humans and animals (Holt et al. 1994) and is therefore of concern in regard to human foodborne illness and is thus the focus of this section. Serotyping is often used to further differentiate *L. monocytogenes* beyond the species level with serotypes 1/2a, 1/2 b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 currently recognized (Nadon et al. 2001; Ryser, 1999).

L. monocytogenes is generally considered a hardy organism unusually tolerant to environmental stresses where strains have been shown to grow at temperatures between 0.4 and 45°C and can survive and grow in foods with water activity and pH values between 0.90 and 0.97 and 4.3 and 10, respectively (Farber and Peterkin 1991; Nolan et al. 1992; Ryser, 2001). *L. monocytogenes* is also capable of growing in salt concentrations of up to 10 % and has been shown to survive for four months in a salt concentration of up to 26 % under refrigeration (Ryser, 2001).

Nature of disease

Listeriosis is the general term for the infection caused by *L. monocytogenes*. Since 1926, when it was first described Murray and colleagues (1926; see Ryser, 1991), scientists have known *L. monocytogenes* infections caused meningoencephalitis and abortion in ruminants such as sheep and cattle (Shank et al. 1996). The first case of human infection was reported by Nyfeldt in 1929 (see Shank et al. 1996), a time when listeriosis was assumed to be caused by direct zoonotic transmission. Although the first documented foodborne outbreak is believed to have occurred in Germany between 1949 and 1957 (Ryser, 1999), *L. monocytogenes* emerged as an important etiologic agent of foodborne disease beginning in the 1980's.

While listeriosis is rare, accounting for only 0.02% of foodborne illness per year, it is responsible for 27.6% of resulting deaths (Mead et al. 1999). Of the estimated 2,500 cases of human listeriosis that occur in the United States annually, 500 result in death (Mead et al. 1999). The high mortality of this pathogen can be attributed to its target population as listeriosis typically affects those with suppressed immune function

including the elderly, fetuses, cancer patients, human immuno deficiency virus (HIV) patients, organ transplant recipients, and individuals receiving corticosteroid therapy. The growing number of people with predisposing factors has increased the size of the at risk population (Schlech, 2000). Additional factors affecting the probability of illness include, but are not limited to, food matrixes and individual strain virulence (CAC, 2000).

The virulence of *L. monocytogenes* centers on its ability to induce phagocytosis by host cells, multiply within the cell cytoplasm and invade adjacent cells. The five genes associated with virulence (*plcA*, *hly*, *mpl*, *actA* and *plcB*) are located in one defined gene cluster under the control of the transcriptional activator, positive regulatory factor A (*prfA*; Sokolovic et al., 1993), encoded by a gene in the same genomic cluster (Kathariou, 2002). These virulence factors are present in all serotypes although their regulation of expression may differ (Kathariou, 2002).

Upon ingestion, surviving cells of *L. monocytogenes* may enter host cells through active ingestion by macrophages. *L. monocytogenes* also adheres to and invades the normally nonphagocytic enterocytes or specialized M cells in Peyer's patches in the small intestine, using surface proteins internalin A (InlA) and internalin B (InlB), depending on the host cell type (Kathariou, 2002). The association with host cell receptors leads to the phosphorylation of host cell proteins and a complex signal transduction cascade resulting in the pathogen-mediated internalization of the bacteria (Kathariou, 2002). Once internalized, *L. monocytogenes* lyses the vacuole through the secretion of a pore forming protein listeriolysin O encoded by *hly*, escaping into the cytoplasm (Kuhn and Goebel,

1999). Here, through the nucleation of host cell derived actin filaments in conjunction with their own ActA protein, cells form an actin tail at one pole providing motility within host cell cytoplasm (Kuhn and Goebel, 1999). Cells reaching the surface of the infected cell induce formation of pseudopod-like structures and are taken up by the neighboring cell. Bacteria, now in the neighboring cell enveloped in a double membrane, escape through the secretion of listeriolysin O and phospholipases (plcA and plcB) and the cycle continues. Dissemination may lead to invasion of target organs including the liver and spleen where most cells are eliminated by macrophages and neutrophils (Kathariou, 2002). T-cell mediated immunity can then detect and destroy cells that have escaped elimination. However, in individuals with compromised immune function, infection of hepatocytes can eventually lead to systemic infection and invasion of secondary targets including the central nervous system, placenta, and fetus (Kathariou, 2002).

Exposure to adverse conditions and stresses such as refrigeration, freezing, heating, acid, salt, dehydration, osmotic stress, as well as exposure to cleaners, sanitizers, and antimicrobial substances during its tenure in foods and/or the environment (Kathariou, 2002) may increase expression of prfA leading to the synthesis of virulence factors for protection (Sokolovic et al., 1993). For example the induction of an ATR following exposure to sublethal pH can provide cross-protection against thermal and osmotic stress among others (O'Driscoll et al. 1996). Acid tolerant mutants have also displayed increased in vivo survival, growth, and virulence. It is therefore possible that repeated exposure to certain stress conditions may select for strains with increased virulence and transmissibility (De Jesus and Whiting, 2003).

Conversely some strains of *L. monocytogenes* display attenuated virulence as a result of the expression of a truncated nonfunctional form of internalin. In a study by Jacquet and others (2004), expression of full length internalin was strongly associated with clinical strains while truncated proteins were strongly associated with isolates from foods. Strains expressing full length proteins represented 95% of strains recovered from non-pregnancy related cases suggesting expression of full length internalin may be necessary for transmission across fetoplacental barrier (Jacquet et al. 2004) and possibly the blood brain barrier although this has yet to be investigated. The observance that serotype 4b and 1/2b strains all expressed full length internalin while strains of serotype 1/2c, rarely associated with clinical isolates, expressed truncated proteins provides epidemiological evidence that the expression of full length internalin is critical in human listeriosis while truncated internalin may be associated with the asymptomatic carriage of *L. monocytogenes* (Jacquet et al. 2004).

Methodology for the detection and isolation of *Listeria* spp. from food and environmental samples

Most standard detection/isolation procedures including those recommended by the ISO, the FDA, and the USDA require 48 hours of enrichment, usually consisting of primary and secondary enrichments for 24 hours each, to allow populations to reach detectable levels followed by plating on selective and/or differential agar media. The accuracy of *Listeria* spp. isolation and detection from food and environmental samples depends greatly upon the enrichment procedures employed (Flanders et al. 1995; Hayes et al. 1991; Hayes et al. 1992; Lund et al. 1991; Noah et al. 1991; Pritchard and Donnelly,

1999; Warburton et al. 1991) where up to 30% of food samples containing *L. monocytogenes* may escape detection (Hayes et al. 1992).

Enrichment procedures. Most enrichment broths contain selective agents to suppress the growth of unwanted microorganisms while not affecting growth of the target organism. However, research has shown that lower maximum population levels are attained when *Listeria* spp. are grown in selective (University of Vermont modified *Listeria* Enrichment; UVM) versus nonselective (BHI) media (Bruhn et al. 2005). Common selective agents used in *Listeria* enrichments include acriflavine to inhibit RNA synthesis and mitochondriogenesis of Gram-positive cocci, nalidixic acid to inhibit DNA synthesis of Gram-negative bacteria and cyclohexamide as an anti-fungal agent (Donnelly, 1999). While concentrations of nalidixic acid between 20 and 40 mg/liter do not appear to effect growth of *L. monocytogenes* (Beumer et al. 1996), acriflavine concentrations of 15 mg/liter can increase the lag time and may inhibit growth of *L. monocytogenes* completely, especially serotype 4b strains (Beumer et al. 1996). The inhibition of serotype 4b strains in food enrichments broths as a result of acriflavine may help explain why 4b is more frequently isolated from listeriosis patients than serotypes isolated from implicated foods (Beumer et al. 1996). As pH decreases, acriflavine can bind to protein in food components, such as cheese, resulting in a decrease of 60% or less of its original concentration thus reducing its inhibitory ability (Beumer et al. 1996). The incorporation of glucose and the limited buffering capacity of *Listeria* enrichment broth (LEB), the FDA recommended enrichment media, results in a decrease in pH during enrichment, thus decreasing the activity of acriflavine resulting in overgrowth of non-

target microflora (Beumer et al. 1996). The ISO enrichment procedure may reduce this inhibitory effect through the use of Half Fraser Broth, which contains half the concentration of acriflavine (12.5mg/liter) of full Fraser broth (FB; 24mg/liter), in primary enrichment.

In addition, selective enrichment media generally do not support the survival and growth of listeriae that may be present in a sublethally injured state resulting from heating, cooling, freezing, and drying or exposure to chemical preservatives such as sodium nitrite, sodium chloride or sorbic, propionic, and acetic acids. The detection of injured listeriae is critical as cells may be capable of repair in food products thus regaining pathogenicity. The use of non-selective enrichment media allows for the repair of injured listeriae and subsequent detection (Donnelly, 1999). It has been suggested that enrichment in non-selective media such as Buffered Peptone Water (BPW) may provide an advantage over UVM in a variety of foods due to the inherent buffering capacity coupled with increased selectivity for *L. monocytogenes* in the absence of selective agents even amidst the presence of large numbers of background flora (Duffy et al. 2001; Walsh et al. 1998). Studies have also shown that shorter enrichment times can also be employed, thus reducing the time needed for detection (Duffy et al. 2001). As with selective media, the use of a non-selective media with more buffering capacity may be especially necessary for the examination of dairy products (Walsh et al. 1998) such as cheese.

Competitive advantage. Certain species of *Listeria* may also have a competitive advantage over others in certain media resulting in false negative results (Beumer et al.

1996; Curiale and Lewus, 1994; MacDonald and Sutherland, 1994; Petran and Swanson, 1993; Ryser et al. 1996). Longer generation times have been demonstrated for *L. monocytogenes* compared to *L. innocua* in BHI (Evanson et al. 1991), FB, UVM and Trypticase Soy Broth (TSB; Curiale and Lewus, 1994). When present together, *L. innocua* may outgrow *L. monocytogenes* in selective media such as LEB (MacDonald and Sutherland, 1994), UVM (Beumer 1996, Curiale and Lewus 1994, Petran 1993) and FB (Curiale and Lewus 1994; Petran and Swanson, 1993). Conversely, some researchers have reported that *L. innocua* has no significant competitive advantage over *L. monocytogenes* when both are present in minced beef samples enriched in either BPW or UVM (Duffy et al. 2001). Based on the conflicting results of the aforementioned studies, it is possible that the individual studies do not reflect strain to strain variation and that a much larger set of isolates needs to be examined to address this topic (Cornu et al. 2002). Variability in competitive fitness among serotypes within species may also help explain why serotype 1/2a strains are isolated from foods and food-processing environments more often serotype 4b strains (Bruhn et al. 2005) which are more commonly associated with foodborne outbreaks as previously mentioned.

It appears as though the competitive overgrowth phenomenon may also extend to the different genetic lineages of *L. monocytogenes*. Bruhn and others (2005) demonstrated that *L. innocua* may outcompete lineage I (but not lineage II) strains in both UVM I and UVM II (Bruhn et al. 2005). Differing lineages may also affect the growth of another when co-inoculated leading to unequal representation of subtypes. Lineage II strains appear to overgrow those of lineage I in both UVM I and II when beginning cell

populations approach 10^6 CFU/ml (Bruhn et al. 2005). This phenomenon may have a detrimental effect on epidemiological investigations as discussed later.

Dual enrichment. The current regulatory procedure for the analysis of red meat, poultry, eggs and environmental samples for *Listeria* was developed by the USDA-FSIS and utilizes UVM as a primary enrichment medium as the selectivity of UVM broth suppresses background flora, allowing for discrimination of low levels of *Listeria* spp. in highly contaminated samples. However, the selective agents in UVM may reduce the extent of recovery and subsequent detection of injured cells (Beumer et al. 1996; Busch and Donnelly, 1992; Ryser et al. 1996). The USDA method (USDA/FSIS, 2005) therefore allows the additional use of *Listeria* Repair Broth (LRB), a medium which allows for the repair and recovery of injured cells over a 5 hour non-selective incubation period to enhance detection (Busch and Donnelly, 1992). While neither enrichment broth is significantly better than the other singly (Ryser et al. 1996), the recovery of *Listeria* spp. from environmental samples can be greatly improved through the use of both LRB and UVM (Flanders et al. 1995; Pritchard and Donnelly, 1999). Furthermore, multiple primary enrichments can be combined in a single secondary enrichment broth, providing the sensitivity of carrying out both procedures individually without the increased workload (Pritchard and Donnelly, 1999).

Agar media plating. Following enrichment, cultures are streaked to one or more agar media selective for the target organism. Current regulatory procedures recommend the use of PALCAM (polymyxin-acriflavine-lithium chloride-ceftazidime-aesculin-mannitol), Oxford and LPM. Detection of *Listeria* via these media is based on the

activity of the enzyme β -D-glucosidase which cleaves esculin in the media to esculetin which in turn reacts with ferric iron to produce brown-black halos surrounding colonies. Since this enzyme is present in all *Listeria* spp., these media are selective for the genus *Listeria* but do not allow for the differentiation between species (Reissbrodt, 2004). Additionally, if significant levels of additional *Listeria* are present, colonies of *L. monocytogenes* may be missed. In turn, the examination of 10 colonies per agar plate has been suggested to assure that identification is based on equal representation of all species that may be present (Ryser et al. 1996), but this practice can be costly, labor intensive and time consuming, especially if further tests are required to differentiate species.

Once isolated, *Listeria* spp. are typically differentiated through hemolysis and CAMP (Christie, Atkins, Munch-Peterson) testing using both *S. aureus* and *Rhodococcus equi* where *L. monocytogenes* is beta hemolytic and with enhanced hemolysis near the *S. aureus* streak but not *R. equi*. Additional testing involves the production of acid from 5% solutions of mannitol, L-rhamnose, and D-xylose (Holt et al. 1994). *L. monocytogenes* produces acid from L-rhamnose but not mannitol or D-xylose (Holt et al. 1994). Although effective, these procedures are relatively time consuming. Recently, chromogenic plating media including, but not limited to, CHROMagar *Listeria* (CHROMagar, Paris France), BCM (Biosynth), ALOA (AES Laboratories, France), and Rapid L'MONO (Sanofi Diagnostic Pasteur, France) have been introduced. These media differentiate virulent strains of *Listeria* (*L. monocytogenes* and *L. ivanovii*) through the use of the lecithinase operon which contains at least two virulence genes *actA* and *plcB*, with the latter encoding phosphatidylcholin-phospholipase C (PC-PLC). Upstream from

hyla, *plcA* encodes phosphatidylinositol-specific phospholipase C (PI-PLC).

CHROMagar *Listeria* utilizes the ability of β -D-glucosidase to cleave a chromogenic substrate to produce turquoise colonies identifying *Listeria* spp. Cleavage of L- α -phosphatidyl-inositol by PI-PLC produces water-insoluble fatty acids around colonies forming a distinct opaque halo zone of precipitation (Reissbrodt, 2004). As a result, the use of chromogenic media can reduce the number of false negative results by distinctly identifying colonies of pathogenic species amongst non-pathogenic background species. Regardless of agar media employed, confirmation of the presence *L. monocytogenes* may take up to 7-8 days. Since this duration may be too long for some industries and applications there is a pressing need for more rapid methods.

Polymerase chain reaction. The use of gene based polymerase chain reaction (PCR) has the potential to reduce pathogen detection time without sacrificing, and possibly enhancing, sensitivity and specificity (Gasnov et al. 2005). *L. monocytogenes* may also escape detection if outnumbered by other *Listeria* spp. such as *L. innocua* on agar plating media (Beumer et al. 1996; Curiale and Lewus, 1994; MacDonald and Sutherland, 1994; Petran and Swanson, 1993; Ryser et al. 1996). The presence of high numbers (10^6 - 10^7 CFU/ml) of other *Listeria* spp., which may mask culture identification of *L. monocytogenes*, does not seem to influence PCR detection (Navas et al. 2006).

The BAX® PCR system (Dupont™ Qualicon BAX® Q7) for screening *Listeria* spp., which generally performs as well or better than standard cultural methods (Norton et al. 2001b, Silbernagel et al. 2004), has been adopted by both the USDA (MLG 8A.03; USDA/FSIS, 2008) and Health Canada (Warburton and Pagotto, 2005) for the screening

of *Listeria* spp. in processing plant environmental samples. The USDA has also approved the use of the automated BAX system for screening meat and poultry samples for *L. monocytogenes*. The procedure still requires 2-stage enrichment (42 to 48 hours) prior to PCR. Following enrichment, bacterial DNA is released via cell lysis, and a targeted DNA sequence is then amplified by PCR. The amplified PCR products are automatically detected within the unit eliminating the need for gel electrophoresis. Although comparatively more expensive, the elimination of more time consuming detection and isolation steps saves a great deal of time. It is important to note that some food (Hoffman and Wiedmann, 2001) and media components (e.g. ferric ammonium chloride; Parameswaran et al. 2003) may inhibit PCR and therefore must be taken into consideration when choosing a suitable detection method.

Previous investigations have employed the BAX system to detect *L. monocytogenes* in environmental samples including those obtained from smoked fish processing plants (Norton et al. 2001b). Although the PCR based system identified more positive samples than the standard cultural method, there were numerous discrepant results attributed to the presence of low population levels of *L. monocytogenes* following enrichment or high levels of background flora. Cultured isolates from BAX false-negative samples tested positive on the BAX system suggesting the false-negative results were not due to the presence of nonreacting isolates. Therefore, false-negative results are likely the result of low population levels achieved through the use of a single enrichment as opposed to a two step procedure which would have likely allowed populations to reach detectable levels (Norton et al. 2001b). The use a single primary enrichment prior to

PCR may result in false negative results when compared to standard cultural procedures (Navas et al. 2006). Furthermore a single primary enrichment may not prevent false positive PCR results from the amplification of DNA from non-viable cells (Navas et al. 2006).

Subtyping

Characterization of isolates beyond the species and subspecies level proves useful in confirming epidemiological links between foods, environments, and cases of human and animal listeriosis (Arimi et al. 1997; Graves et al. 1994). Subtyping has been employed to track strains contaminating food processing plants (Autio et al. 1999; Hoffman et al. 2003; Lappi et al. 2004; Norton et al. 2001a; Sutherland and Porritt 1996; Thimothe et al. 2004; Wulff et al. 2006) and farm environments (Arimi et al. 1997; Nightingale et al. 2004) providing an effective means of monitoring the persistence of resident populations and facilitating a better understanding of pathogen ecology (Norton et al. 2001b). Improved understanding of contamination sources can aid processors in developing more effective cleaning and disinfection programs aimed at the elimination of resident populations while minimizing colonization (Norton et al. 2001b) in an effort to prevent cross contamination (Wulff et al. 2006). Both phenotyping and molecular typing methods have been utilized to subtype strains of *L. monocytogenes* with the ideal method providing high discriminatory power, typeability, reproducibility and automation (Wagner and Allerberger, 2003).

Serotyping was one of the first phenotyping methods employed and while thirteen serotypes of *L. monocytogenes* are recognized as previously described, only three (1/2a,

1/2b and 4b) are generally considered important from a public health standpoint (Farber and Peterkin, 1991). It was observed early on that sporadic human listeriosis cases were typically associated with serotypes 4b and 1/2b (McLauchlin, 1990a) while outbreaks were associated with serotype 4b (Wiedmann, 2002) including those associated with cheese (Pintado et al. 2005). Although clinical isolates represent a heterogeneous group, isolates do not always match those of food isolates (Gilbreth et al. 2005; Gray et al. 2004; Lukinmaa et al. 2004). For example, serotype 4b strains which are thought to cause more than 50% of listeriosis cases worldwide (Schuchat et al. 1991), are rarely isolated from foods (Gilbreth et al. 2005). It is hypothesized that those serotypes rarely recovered from patients may pose a relatively low risk for human listeriosis (Kathariou, 2002) and that serotypes differ in virulence potential (Gray et al. 2004; Jacquet et al. 2004).

In general isolates obtained from foods are more commonly serotype 1/2 (Farber and Peterkin, 1991; Guerra et al. 2001; Jacquet et al. 2004; Lukinmaa et al. 2004; Rorvik, 1995), and more specifically 1/2a (Gilbreth et al. 2005). Serotype 1/2a strains have been isolated on the dairy farm from such sources as feed, silage and feces among other environmental samples (Borucki et al. 2004) and are also frequently isolated from RTE foods and food processing environments (Kathariou, 2002). Some researchers have reported that serogroup 1/2 strains appear to dominate isolates from soft cheese (Pini and Gilbert, 1988; Loncarevic et al. 1995) while others report a predominance of 4b followed by 1/2 (Pintado et al. 2005).

Molecular subtyping. Originally serotyping was used as an epidemiologic tool but proved to be inadequate due to poor discriminatory power (Schuchat et al. 1991).

Bacteriophage typing was slightly more discriminatory and could be applied to a large set of isolates, but lacked typeability. Molecular subtyping, however, has the advantage of high discriminating ability coupled with 100% typeability (Graves et al. 1994). Several molecular typing methods have also been employed including but not limited to, multi-locus enzyme electrophoresis (MEE), multi-locus virulence sequence typing (MVLST), multiple-locus variable-number tandem repeat analysis (MLVA), restriction enzyme analysis, pulsed field gel electrophoresis (PFGE), single nucleotide polymorphism (SNP) genotyping, terminal fragment length polymorphism (T-RFLP) analysis, and ribotyping (Graves et al. 1999; Schuchat et al. 1991). In an early study by Graves and others (1994) it was concluded that ribotyping, similar to MEE, was an acceptable method for subtyping *L. monocytogenes* with the possible exception of serotypes 1/2b and 4b.

Ribotyping. The subtyping of *L. monocytogenes* isolates via ribotyping became more common following the introduction of the automated Riboprinter® Microbial Characterization System by DuPont Qualicon in the mid 1990s. In the automated system, restriction endonuclease *EcoRI* (Qualicon) or *PvuII* (Qualicon) DNA fragments are separated by size using electrophoresis and analyzed using a modified southern hybridization blotting technique. The DNA is hybridized with a labeled *E. coli* rRNA operon probe with resulting bands detected using a chemiluminescent substrate. Images are acquired with a charge coupled device camera and processed using the RiboExplorer® software which normalizes fragment pattern data for band density and proximity with bands normalized to a standard marker set. The resulting patterns are automatically characterized and identified using similarity measurements to previously

run isolates and reference patterns provided in the RiboPrinter database. DuPont IDs (DUP-IDs) are assigned to isolates when sample patterns match a pattern in the database with a similarity >0.86. Using a proprietary algorithm, genetically related isolates within a similarity range >0.93, are combined to form a ribogroup (Bruce, 1996).

One possible drawback of this is that ribotype patterns are assigned with lesser discretion when only a small number of patterns exist for that type. The more patterns a database groups, the more discriminatory it becomes. This is a drawback that may result in the grouping together of isolates that are not as similar as others (Lukinmaa et al. 2004). One major downfall of the automated system is that at times an assigned DUP-ID can include more than one distinct ribotype pattern, typically due to the presence of an additional single weak band. Researchers at Cornell University have established a database of distinct subsets within DUP-IDs assigned alphabetically. These patterns with assigned alphabetical subsets allow for comparison to the majority of data in the scientific literature. If the Cornell data is not available, RiboExplorer files can be entered into the public database PathogenTracker 2.0 (www.pathogentracker.net) and compared to patterns contained therein, albeit with varying results. Strains can then be assigned with the additional alphabetized letter (e.g., DUP-1042B) through a comparison with similar patterns identified in PathogenTracker 2.0.

It is known that the discriminatory power of automated ribotyping using *EcoRI* is lower than that of PFGE, especially in the discrimination of serotype 4b strains important to epidemiologic investigations (Lukinmaa et al. 2004; Swaminathan et al. 1996). Discriminatory ability of ribotyping can be improved, including that of 4b strains,

through the use additional restriction enzymes such as *PvuII* (Aarnisalo et al., 2003; De Cesare et al. 2001). Automated ribotyping is the preferred subtyping method for large data sets because it is fast, easy to perform, highly standardized, objective, and labor-saving (Lukinmaa et al. 2004). More importantly it is a sensitive and reproducible allowing for comparative analyses between laboratories through large data sets found in numerous publications and public databases (i.e. PathogenTracker).

Automated ribotyping has been used to confirm epidemiological links between foods, environments, and cases of human and animal listeriosis (Aarnisalo et al. 2003, Allerberger and Fritschel, 1999; Dalton et al. 1997; Felicio et al. 2007; Graves et al. 1994; Jaradat et al. 2002; Jeffers et al. 2001; Lukinmaa et al. 2004; Manfreda et al. 2005; Norton et al. 2001b; Suihko et al. 2002; Swaminathan et al. 1996). Ribotyping has also been employed to track strains contaminating farm environments (Arimi et al. 1997; Nightingale et al. 2004) and food processing plants (De Cesare et al. 2007; Ho et al. 2007; Kabuki et al. 2004; Wulff et al. 2006) including those manufacturing cheese (De Cesare et al. 2007; Ho et al. 2007).

More recently ribotypes have been used to predict serotype classifications (De Cesare et al. 2007; Nadon et al. 2001). Currently isolates characterized by the Riboprinter as DUP-IDs often contain more than one serotype regardless of lineage whereby only eighteen ribotypes appear to be predictive of serotype (Nadon et al. 2001). Such predictions can be improved through the use of multiple restriction enzymes including *PvuII* (Nadon et al. 2001).

Genetic lineage

Using the molecular subtyping techniques including PFGE (Brosch et al. 1994), MEE (Graves et al. 1994) and ribotyping (Graves et al. 1994), strains of *L. monocytogenes* were originally grouped into two primary subgroups each containing similar serotypes. Soon thereafter, ribotyping and allelic analysis further subdivided strains of *L. monocytogenes* into three distinct lineages deemed I, II, and III (Wiedmann et al. 1997). While lineages I and II correspond to the primary subdivisions identified by PFGE and MEE, lineage III strains represent a distinct subset of *L. monocytogenes* (Nadon et al. 2001). These lineages also contain predicted serovar clusters, where serotypes 1/2b, 4b, 3b, and 3c group into lineage I, serotypes 1/2a, 1/2c and 3a group into lineage II, and serotypes 4a, 4b, and 4c group into lineage III. A correlation also exists between lineage and single antigens where lineages II and III contain flagellar antigens a and c, while lineage I mostly contains antigen b (Nadon et al. 2001). More recently, MVLST also identified the three known lineages with congruent serotypes (Chen et al. 2007).

Although lineage does not appear to be associated with either clinical manifestations or disease outcome (Sauders et al. 2006), they do appear to differ in their association with specific hosts and environments as well as pathogenic potential (Chen et al. 2006; Gray et al. 2004; Jeffers et al. 2001; Wiedmann et al. 1997). In general lineage I strains are more commonly associated with human clinical isolates (Sauders et al. 2006) than those in lineage II (Norton et al. 2001a) which are typically associated with isolates from food and food processing environments (Gray et al. 2004; Norton et al. 2001a;

Sauders et al. 2004; Suihko et al. 2002; Wulff et al. 2006). This observation could be an artifact of the differences in isolation methodology between food, clinical, and environmental samples, including enrichment procedures that may favor the isolation of specific lineages and therefore bias associations. Additionally, strains within lineages are not wholly homogenous thus complicating attempts at correlating lineage to thermotolerance (De Jesus and Whiting, 2003) and the ability to form biofilms (Borucki et al. 2003; Djordjevic et al. 2002) where any differences observed seem to be strain specific as opposed to lineage specific.

Lineage I. Serotype 4b strains of lineage I are the predominant strains isolated from human listeriosis cases and outbreaks worldwide (Gray et al. 2004; Jeffers et al. 2001; Sauders et al. 2006; Wiedmann, 2002) including the most highly publicized outbreaks over the past 20 years in both Europe and the U.S.(Kathariou, 2002). As a result, nearly all human epidemic isolates, or epidemic clones (EC), cluster into lineage I (Jeffers et al. 2001; Kathariou, 2002). These strains also tend to produce significantly larger plaques in cell culture assays than strains of other lineages (Gray et al. 2004; Norton et al. 2001a, Wiedmann et al. 1997) suggesting they have a higher likelihood of causing disease (Gray et al. 2004). Although lineage I strains are highly associated with human listeriosis (Gray et al. 2004), they are rarely isolated from food and food processing environments (Gray et al. 2004; Norton et al. 2001a) as a possible result of reduced environmental survival and growth capabilities (Chen et al. 2006).

Lineage II. Lineage II strains, including persistent ribotypes (Norton et al. 2001a) are more commonly associated with isolates from foods (including fresh soft

cheeses and soft ripened cheeses; Chen et al. 2006) and food processing environments (Gray et al. 2004; Norton et al. 2001a; Sauders et al. 2004; Suihko et al. 2002; Wulff et al. 2006) and are comparatively underrepresented in human listeriosis cases (Norton et al. 2001a). Therefore it is believed that certain subtypes may exhibit enhanced environmental survival and growth capabilities (Chen et al. 2006), such as a shorter lag phase in cold conditions (7°C) than strains from other lineages (De Jesus and Whiting, 2003). As a possible result of the stresses encountered in these environments it has been suggested that this lineage may be very young and/or under strong selective pressure (Kathariou, 2002).

If present in foods, lineage II strains appear to exist at higher concentrations than those of lineage I, yet have a lower log average probability of causing listeriosis (Chen et al. 2006). It has been suggested that this reduced ability to cause human disease is the result of attenuated virulence due to pre-mature *inlA* stop codons (PMSC; Sauders et al. 2006). This may help explain why the lineage II strains are underrepresented in human clinical isolates when compared to their prevalence in food (Sauders et al. 2006). For example, strains of DUP-1046B, which are rarely isolated from human clinical samples, may have an impaired ability to spread from cell to cell or infect mammalian cells based on their inability to form plaques in cell culture plaque assays (Norton et al. 2001a).

Although lineage II contains isolates related to sporadic cases, albeit less than lineage I, few have been associated with foodborne epidemics (Wiedmann et al. 1997). Lineage II does, however, contain EC III (Kathariou, 2002), a ribotype linked to a major

outbreak of listeriosis involving turkey deli meat as well as DUP-1030, a strain linked to an outbreak of listeriosis that occurred in Carlisle England in 1981 (Jeffers et al. 2001).

Lineage III. To date strains of lineage III have yet to be linked to humans or foods involved in an outbreak of listeriosis (De Jesus and Whiting, 2003). Isolated more frequently from animals than humans (Jeffers et al. 2001), it has been suggested that lineage III strains are particularly virulent to animals but rarely cause human disease (Wiedmann et al. 1997) and may therefore have host specificity for non-primate mammals with limited virulence in humans (Jeffers et al. 2001). It has also been hypothesized that some lineage III strains carrying *actA* allele 3 have attenuated virulence in humans (Jeffers et al. 2001). It is also possible that the lack of human cases may also result from limited human exposure (Sauders et al. 2006) as heat inactivation studies have shown that on average lineage III strains are the most heat labile which may also explain the limited association with foods (Gray et al. 2004).

Epidemic clones

Listeriosis outbreaks are typically caused by a small portion of *L. monocytogenes* strains (Chen et al. 2007) with most major outbreaks attributed to a small number of ECs, defined as isolates that descended from a common ancestor which possess similar genetic characteristics. ECs are thought to possess an enhanced ability to colonize food processing facilities (Kathariou, 2002) as well as increased virulence (Tompkin, 2002). ECs may also have a lower infectious dose than other strains of *L. monocytogenes* (Chen et al. 2007) further contributing to the increased likelihood of causing listeriosis.

Based on the association with human listeriosis clusters, human epidemic isolates of *L. monocytogenes* were originally thought to represent two unique clones (Piffaretti et al. 1989) equivalent to ribotypes DUP-1038 and DUP-1042 referred to as ECI. More recently, however, three additional epidemic clones were identified bringing the total to four. ECI and Ia (as defined by Kathariou, 2002), serotype 4b clusters, are represented by strains of ribotypes DUP-1038B and DUP-1042B, respectively (Kathariou, 2002), while strains of ribotype DUP-1044A and DUP-1053A are referred to as ECII and ECIII, respectively (Kathariou, 2002). It is likely that previously or newly identified outbreak strains will be implicated in future outbreaks of listeriosis making the tracking of said strains critical in the investigation and prevention of listeriosis outbreaks (Chen et al. 2007).

Epidemic clone I. ECs within lineage I appear to cause a large number of human listeriosis outbreaks as well as cases of sporadic illness (Gray et al. 2004; Sauders et al. 2004; Wiedmann et al. 1997) as the possible result of enhanced transmissibility and/or virulence (Chen et al. 2006; Sauders et al. 2006) or host adaptation (Chen et al. 2006). ECI, originally consisting of DUP-1042 and DUP-1038 strains, are responsible for numerous outbreaks of epidemic listeriosis (Jeffers et al. 2001). In 2001, the discriminatory ability of ribotyping was improved through the use of multiple restriction endonucleases (De Cesare et al. 2001) resulting in the differentiation of four ribotypes among DUP-1042 isolates and three ribotypes among those of DUP-1038 (De Cesare et al. 2001) each assigned additional letter designations (i.e. DUP-1042B).

ECI DUP-1038 strains are significantly more common among epidemic isolates than human sporadic isolates and have been associated with major outbreaks attributed to cole slaw in Nova Scotia, pork tongue in France, as well as cheese in California and Switzerland (Jeffers et al. 2001). Early investigations suggested that DUP-1038 had a unique potential to cause foodborne human disease as a result of increased virulence or ability to survive and grow in foods (Piffaretti et al. 1989). While higher than average plaque formation observed during in vitro plaque assays supports increased virulence, the significant association of DUP-1038B with human isolates compared to those from food (Gray et al. 2004) suggests survival in foods may not be the critical factor. Human isolates also display higher infectivity than those from food (Gray et al. 2004).

In 2001, Jeffers and colleagues linked strain DUP-1042 to outbreaks involving raw vegetables in Boston, pate in the U.K., ice cream in Philadelphia, and pasteurized milk in Massachusetts. Although DUP-1042 is linked to major outbreaks, strains are not significantly more common among human isolates compared to those from animals suggesting the strain may be more common as opposed to uniquely pathogenic (Jeffers et al. 2001). DUP-1042 was later divided into unique subgroups based on slight variations in fragment patterns. Using the new subdivisions, Gray and others (2004) found that DUP-1042B was more common among human than food isolates than other DUP-1042 strains. This subdivision also produced larger plaque areas than other ribotypes (Gray et al. 2001). It was soon realized that this subdivision (DUP-1042B) was specifically responsible for the aforementioned outbreaks attributed to DUP-1042 and was more specifically designated as an ECI strain. DUP-1042B was later determined to be clonally

different than DUP-1038B and was renamed ECIIa by Kathariou and others in 2002. Recently, more discriminatory subtyping via MVLST solely grouped isolates from the vegetable and pate outbreaks together as ECIIa (Chen et al. 2007). Although other strains of DUP-1042B have been associated with outbreaks including Mexican-style cheese in North Carolina, ice cream in Philadelphia, and pasteurized milk in Massachusetts, these strains are not considered to be ECIIa. Rather these strains represent single outbreak strains or outbreak clones (Chen et al. 2007). As a result of these observations, Chen and others (2007) renamed ECIIa as ECIV, which now only includes strains of DUP-1042B associated with the vegetable and pate outbreaks.

Epidemic clone II. ECII, represented by strains of DUP-1044A, was recognized in 1999 following a prolonged multistate outbreak attributed to contaminated hot dogs (Graves et al. 2005) and later linked to the 2002 multistate outbreak of listeriosis associated with turkey deli meat (Chen et al. 2005). It is possible that ECII strains possess an enhanced ability to survive and persist under environmental conditions (Norton et al. 2001a) which may have played a role in the aforementioned outbreaks. ECII serotype 4b strains differ from other serotype 4b strains in that conserved DNA fragments adjacent to *inlA* are either absent or markedly divergent (Evans et al. 2004).

Epidemic clone III. In 2000, a multistate outbreak of listeriosis was attributed to turkey deli meat products contaminated with *L. monocytogenes* DUP-1053A. The genotype and serotype (1/2a; Olsen, 2005) of *L. monocytogenes* isolates matched those previously isolated from an outbreak associated with turkey hot dogs in 1988 (CDC, 1989). The same products in both outbreaks were manufactured in the same food

processing facility suggesting that the strain (DUP-1053A) persisted in the plant over the 12 years (Kathariou, 2001) and was thus deemed ECIII. Despite these major outbreaks ECIII is less prevalent among human cases and does not seem to have the same infectivity as the other epidemic clones (Gray et al. 2004).

Outbreaks of foodborne listeriosis and regulatory history

The first documented foodborne outbreak of listeriosis is believed to have occurred in Germany between 1945 and 1952 (Ryser, 1999) prompted by upwards of 100 stillbirths documented at a single clinic in Halle, Germany (Norton and Braden, 2007). Following investigation, the isolates obtained from the milk of a cow with atypical mastitis and still born infants of a mother who had consumed the raw milk of that cow were of the same serotype (Norton and Braden, 2007). Although this case represents the first documented outbreak, *L. monocytogenes* became a major foodborne concern beginning in 1981 following 41 cases of listeriosis (34 perinatal and 7 adult) reported in Canada. Of these, 15 perinatal (44%) and 2 (29%) adult deaths were reported resulting in a 41% fatality rate. Case control studies and traceback investigations identified the outbreak source as coleslaw produced from cabbage harvested from fields fertilized with untreated sheep manure obtained from a farm with a history of ovine listeriosis (Ryser, 1991; Schlech, 1983).

Dairy products were implicated again in 1983 when 49 cases of *L. monocytogenes* infection, with a case fatality rate of 29%, were attributed to the consumption of pasteurized milk in Massachusetts (Fleming et al. 1985). Soon thereafter in 1985, a large epidemic resulting in 142 cases of illness and 88 deaths occurred in Los Angeles County,

California, bringing *L. monocytogenes* as a foodborne pathogen to the forefront of public health and regulatory concern (Norton and Braden, 2007). Infections, a majority of which resulted in death (63%), occurred predominantly in perinatal Hispanic women. Of the non-pregnant cases, 98% occurred in individuals with compromised immune function as a result of cancer, AIDS, diabetes, steroid use or age thus providing an early identification of the at risk population. Following two case control studies among perinatal listeriosis patients, a specific brand (Jalisco Mexican Products Inc.) of soft, unripened Mexican-style cheese (queso fresco) was implicated as the likely vehicle of infection (Ryser, 1999). *L. monocytogenes* serotype 4b isolates obtained from cheese collected from patients' homes, unopened packages purchased at supermarkets and from cheese recalled from 26 states and outlying areas were found to be of the same phage type (Norton and Braden, 2007). The milk at this plant was reportedly pasteurized on site but the presence of high levels of the enzyme alkaline phosphatase in cheese samples produced over six months indicated that the process was somehow deficient. Records indicating that the quantity of raw milk coming in to the plant exceeded the capacity of the pasteurizer suggests that the milk used for cheesemaking was either improperly pasteurized as a result of being over capacity or had raw milk added directly to it (Linnan et al. 1988; Norton and Braden, 2007). In response to these outbreaks, the FDA revised the Compliance Policy Guides for pathogens in dairy products (CPG 7106.08); a document that provides guidance for initiating legal action in cases involving products found to be improperly pasteurized, contaminated with pathogenic microorganisms, or prepared and packed under unsanitary conditions (USFDA/ORA, 2005).

In addition to the recent outbreaks, it was soon concluded that foodborne transmission may account for a substantial portion of sporadic listeriosis cases as well (Schuchat et al. 1992) marking the beginning of what was referred to as “*Listeria* hysteria”. In 1985, based on the known characteristics of the microorganism and the disease, the FDA established a policy of "zero-tolerance" for *L. monocytogenes* in RTE foods where the detection of any *L. monocytogenes* in either of two 25-gram samples of a food using the FDA detection method renders the food adulterated as defined by the Federal Food, Drug, and Cosmetic Act, 21 U.S.C. 342 sec. 402(a)(1) (Shank et al., 1996).

Cases of listeriosis attributed to the consumption of dairy products were soon observed outside the U.S. In an independent effort, following the Jalisco cheese outbreak and the subsequent FDA action in the U.S., Swiss health officials began a surveillance effort to determine the prevalence of *L. monocytogenes* in cheese and other dairy products in Switzerland. Officials soon isolated *L. monocytogenes* from samples of a soft, washed rind cheese, Vacherin Mont D’Or, which is primarily manufactured in and around the Swiss Canton of Vaud in the winter months (Ryser, 1999). Interestingly, medical facilities in Vaud had previously noticed an abnormal increase in cases of listeriosis beginning in January 1983. Similar increases in cases during winter months were observed over the course of the next few years suggesting a common, yet unidentified source. It wasn’t until further investigation by Swiss health officials revealed that two of the phage types unique to the cheese matched those of the patient isolates observed in the ongoing outbreak. A third case-control study conducted in 1987 further implicated the cheese as the likely source of infection. In the end, between 1983

and 1987, 122 cases of listeriosis including 33 deaths were attributed to the consumption of contaminated Vacherin Mont D'Or (Lunden et al., 2004). Although traditionally produced from raw milk, the 40 producers manufacturing this cheese in Switzerland converted to the use of pasteurized milk in 1983, prior to the outbreak suggesting post-pasteurization contamination (Norton and Braden, 2007). In support of this theory, it was reported that cheeses were commonly transferred between aging caves and that wooden hoops were shared and returned to different producers without prior disinfection. Follow up investigations revealed that half of the cellars were contaminated with one or both of the outbreak phage types. Following this discovery, equipment was replaced with those which are more easily cleaned and rigorous cleaning and sanitizing procedures were implemented effectively ending the outbreak (Norton and Braden, 2007).

Laboratory-based surveillance in Denmark identified a specific phage type of *L. monocytogenes* as being responsible for an outbreak which spanned from March of 1989 to December of 1990, resulting in 26 cases of listeriosis and six deaths. Although never microbiologically confirmed, food history questionnaires from the original case-control study indicated a link between the consumption of a Danish blue cheese and listeriosis cases (Norton and Braden, 2007). Around this same time two sporadic cases of listeriosis were also reported in the United Kingdom attributed to the consumption of soft cheese (Bannister, 1987; McClaughlin et al., 1990).

Routine surveillance for *L. monocytogenes* in humans and food in France, which began in 1987, identified a cluster of listeriosis cases caused by an unusual phage type that was also isolated from surveillance samples of Brie de Meaux, a French surface-

mold-ripened soft cheese made from raw milk (Ryser, 1999). Using pulsed field gel electrophoresis (PFGE) investigators were able to match many of the patient and food isolates more specifically, pinpointing a specific batch of the cheese. By 1995, 33 cases resulting in 11 deaths were attributed to this outbreak (Lunden et al., 2004). Although the same strain was isolated from others types of cheese ripened at the same facility it is unclear as to whether the raw milk or a contaminated environment served as the initial source of contamination (Ryser, 1999). Two years later, the French National Research Center (NRC) identified 14 cases of listeriosis over a four month period traced to Pont L'Eveque, another soft, washed rind cheese produced in Normandy. The cheese, which was also manufactured from raw milk, reportedly contained levels of *L. monocytogenes* exceeding 1000 CFU per gram (Ryser, 1999).

In 1988, an Informal Working Group on Foodborne Listeriosis convened by the World Health Organization (WHO Working Group, 1988) concluded that "*Listeria monocytogenes* should be considered as an environmental contaminant, whose primary means of transmission to humans is through contamination of foodstuffs at any point in the food chain". To determine the incidence of listeriosis and identify risk factors for disease, during 1988-1990, CDC collaborated with investigators in four states to conduct active laboratory-based surveillance and special studies in a population of more than 18 million U.S. residents (Pinner et al. 1992; Schuchat et al. 1992). Results indicated that a large proportion of sporadic cases of listeriosis were associated with soft cheese, undercooked poultry, hot dogs (not thoroughly reheated) and foods from delicatessen counters (Anderson et al. 1992). Both the FDA and FSIS implemented monitoring

programs for *L. monocytogenes* to help government and industry identify the causes of contamination in processing plants and to make changes to reduce contamination. The FDA programs focused primarily on soft cheeses and other dairy products while FSIS focused on selected cooked and RTE meat or poultry products. To further reduce the risk of foodborne illness associated with the consumption of meat and poultry products, FSIS issued the Pathogen Reduction-Hazard Analysis and Critical Control Point (HACCP) Systems final rule (USDA/FSIS, 1996). With this regulation, FSIS requires federally inspected establishments to take preventive and corrective measures at each stage of the food production process where food safety hazards can occur.

Pasteurized milk was again implicated in an outbreak of listeriosis in July of 1994. This time an outbreak of gastroenteritis and fever among people who ate at a picnic at a cow show was traced to the consumption of pasteurized chocolate milk. Post-pasteurization contamination due to poor sanitation practices at the dairy followed by extensive temperature abuse allowed levels of *L. monocytogenes* in unopened containers to reach 8 to 9 log CFU per milliliter (Dalton et al. 1997).

Following numerous outbreaks attributed to dairy products, attention soon turned to RTE meat and poultry products. From August 1998 through January of 1999, consumption of hot dogs contaminated with a rare strain of *L. monocytogenes* from a single manufacturer, resulted in 108 illnesses in 24 states causing 14 deaths and four miscarriages or stillbirths (Graves et al. 2005). Then from May through November of 2000, turkey deli meat was implicated in 29 cases of listeriosis (8 perinatal) identified in 10 states (Hurd et al. 2000; Olsen 2005).

Mexican-style cheese was once again implicated in an outbreak of listeriosis first identified as a small cluster of perinatal cases in North Carolina in 2000. In the end 13 people, including 11 pregnant females, were infected with *L. monocytogenes* resulting in five still births. Case-control studies implicated homemade raw milk Mexican-style soft cheese purchased in unlabelled packages from small Hispanic markets, street vendors, or through door to door sales. The outbreak strain was isolated from cheese obtained from a patient's home and two area grocery stores as well as the raw milk from the bulk tank of manufacturing grade dairy who admitted to illegally selling the raw milk. The source of the raw milk contamination was never identified as all individual animal samples were negative (McDonald et al., 2005). Mexican-style cheese produced in Mexico and illegally distributed in Texas resulted in yet another outbreak of listeriosis in 2003. Five of the six women affected reported consuming queso fresco cheese which was made from raw milk in Mexico and sold at flea markets and unlicensed street vendors in the U.S. (Norton and Braden, 2007).

In response to multiple outbreaks and in effort to reduce the incidence of listeriosis associated with consumption of RTE foods, HHS and USDA issued the *Listeria* Action Plan calling for an increase in the education of consumers and health care providers. The plan also suggested the review and revision of enforcement and regulatory strategies, including microbial product sampling and enhanced disease surveillance, outbreak response, and research (USDA, 2000). However, later in 2002, 54 illnesses across nine states resulting in eight deaths (three fetal) were again linked to the consumption of turkey deli meat between the months of July and November. The

outbreak strain was isolated from the environment of one processing plant and in turkey products from another who, together, recalled >30 million pounds of products. In response FSIS issued Directive 10,240.3: "Microbial Sampling of Ready-to-Eat (RTE) Products for the FSIS Verification Testing Program" which outlines a *L. monocytogenes* control and testing program for RTE meat and poultry processing plants (Gottlieb et al. 2006).

Although outbreaks can range from short duration and geographically defined such as occurred with butter in Finland (Lyytikäinen et al. 2000), to ongoing and widespread as witnessed in the multistate turkey deli meat outbreak (Olsen et al. 2005), researchers now believe that most cases of infection may be sporadic and not part of large outbreaks (Schlech, 2000; Varma et al. 2007). However, since the overall incidence of disease is low, a large outbreak across numerous states or countries could affect a large number of people as a whole without alerting public health officials. These multistate outbreaks are difficult to detect making cases appear to be sporadic and localized as opposed to part of a larger issue. Data from Sauders and colleagues (2003) also demonstrates that a large proportion of cases of human listeriosis represent subtype clusters, some or all of which may represent common source outbreaks. The increase in molecular subtype surveillance, such as PulseNet in the U.S., may soon reveal that outbreaks and common source clusters are more common (Sauders et al. 2006).

Incidence in milk and milk products.

L. monocytogenes is commonly isolated from numerous food categories including meat, poultry, fish and dairy products resulting from the contamination of raw materials,

environmental contamination during or after processing, at retail, or even in the consumer home. It has even been suggested that processed, rather than raw foods, may be more frequently contaminated by all species of *Listeria* (Guerra et al. 2001).

Combined data from numerous surveys conducted worldwide suggests that approximately 2.2% to 3.8% of raw cow's milk is likely to contain *L. monocytogenes* (Farber and Peterkin 1991; Ryser, 1991). While some surveys have failed to detect any *L. monocytogenes* in bulk tank milk (Lovett et. al. 1987), the incidence rate for cow's milk reported in surveys conducted throughout the U.S. range from 2.8 to 7% (Jayarao et. al. 2006; Jayarao and Henning, 2001; Lovett. et. al. 1987; Muraoka 2003; Rohrbach et. al. 1992; Van Kessel et. al. 2004). Outside the U.S., reported prevalence ranges from 1 to 5.3% throughout Europe (Gaya et. al. 1998; Greenwood et. al 1991; Harvey and Gilmour 1992; Rea et. al. 1992; Sanaa et. al. 1993; Waak et. al. 2002) and Ontario, Canada (Steele et. al. 1997). Higher incidence rates up to 33.3% (Harvey and Gilmour, 1992) have been reported for dairy silo milk attributed to the longer storage time and the commingling of milk from numerous producers (Waak et. al. 2002). While both goats (Abou-Eleinin et. al. 2000) and sheep (Rodriguez et. al. 1994) are known carriers of *L. monocytogenes*, contamination of small ruminant milk appears to be less common, with average incidences of 1.6% and 2.4% reported for sheep and goat milk, respectively (Gaya et. al. 1996; Rodriguez et. al. 1994, Ryser, 1991). According to Ryser (1991) approximately 0.67% of pasteurized milk and 5% of frozen dairy products manufactured in the U.S. could contain *L. monocytogenes* (Ryser, 1991) as the result of post-pasteurization contamination.

According to a review by Ryser (2007), FDA records revealed that the presence of *L. monocytogenes* was confirmed in 12 of 658 (1.82%) domestic cheese samples analyzed in 1986. The following year in a sampling of domestic aged natural cheese manufactured from raw milk, only one of 181 samples was positive for *L. monocytogenes*. These data could represent a limited incidence of *L. monocytogenes* in cheese or could simply be an artifact of the extensive recalls of contaminated cheese in the U.S. Similarly, intensive FDA surveillance has prompted recalls of imported cheese. Based on the results of multiple surveys Ryser (2007) reports that contamination of French cheeses is likely to be <10% with 108 of 2425 (4.5%) cheeses surveyed yielding *L. monocytogenes* with soft cheeses manufactured from raw milk 5.7 times more likely to be contaminated as their pasteurized milk counterparts. Similar results were reported for Germany (4.4%), Italy (3%) and Switzerland (4.9%), with contamination most commonly observed in soft followed by semi-soft and hard varieties.

Environmental incidence

Farm environment. *L. monocytogenes* occurs widely in dairy farm environments (Nightingale et al. 2004, Borucki et al. 2004) although prevalence on sheep and goat farms may be lower than that of cows (Nightingale et al. 2004). Contamination of the farm environment with *L. monocytogenes* likely begins with animals initially exposed via contaminated feedstuffs (Nightingale et al. 2004) which are likely contaminated through soil (Nightingale et al 2004). Feedstuffs such as stock straw (Ueno et al. 1996) and improperly fermented (pH > 5) silage (hay, grass or corn), which can harbor a diverse group of ribotypes (Borucki et al. 2004; Ryser et al. 1997; Wiedmann et

al. 1996), has long been considered an important source of *L. monocytogenes* on the farm and in cases of listeriosis in ruminants (Ryser, 1991; Wiedmann et al. 1994, 1996). Once ingested, *L. monocytogenes* can be shed in the feces of healthy dairy goat, sheep (Nightingale et al. 2004) and cows for months to several years (Lovett et al. 1987) dispersing a diverse population of subtypes, including bovine clinical isolates (Borucki et al. 2004) into the soil and farm environment (Nightingale et al. 2004). Fecal shedding can also contaminate water tanks, milk socks and silage through the spreading of manure as fertilizer (Borucki et al. 2005) further contributing to a contamination cycle of fecal spread and oral infection (Borucki et al. 2004).

Food processing plants. Listeriae, including *L. monocytogenes*, are common contaminants of food processing facilities. Some strains of *L. monocytogenes* have been shown to persist in processing environments (Borucki et al. 2004; Ho et al. 2007; Hoffman et al. 2003; Holah et al. 2002; Lappi et al. 2004; Norton et al. 2001b; Sutherland and Porritt, 1996; Thimothe et al. 2004; Wulff et al. 2006) possibly as a result of physical adaptation (surface attachment, biofilm formation, etc.) to the range of environmental conditions encountered (low temperature, wide pH range, fluctuating moisture, etc; Kathariou, 2002). Persistent strains have also been shown to be significantly more adept to form biofilms than sporadically isolated strains (Borucki et al. 2003). These biofilms typically formed in joints, valves, gaskets, as well as in the pits and cracks of corroded surfaces, are more resistant to effects of adverse environments (Lee Wong, 1998). Persistent strains may therefore constitute part of the resident microflora not eliminated by current cleaning and sanitation of these potential niches (Norton et al. 2001b) which

may indicate that prerequisite programs such as sanitation standard operating procedures (SSOPs) and good manufacturing practices are inadequate (Wulff et al. 2006). It is also possible that specific conditions in a plant select for resident strains while inhibiting others as a result of effective competition against other bacteria including other strains of *Listeria* or by unknown mechanisms such as phage resistance, bacteriocin production and resistance to disinfectants (Kathariou, 2002).

Once strains of *L. monocytogenes* have become established, the environment can serve as a source of food product contamination (Holah et al. 2002; Jacquet et al. 1993; Kozak et al. 1996; Lee Wong, 1998; Norton et al. 2001b; Thimothe et al. 2004) and possibly human listeriosis (Borucki et al. 2004; Harvey and Gilmour, 1994; Linnan et al. 1998; McClaughlin et al. 1990; Ryser, 1999). The differential ability to persist in certain environments as a result of surface adherence and biofilm formation may play a role in the ability of a strain to cause a foodborne illness (Borucki et al. 2003) and may also be a contributing factor to clusters and outbreaks of human listeriosis. The consumption of RTE foods contaminated as the result of post-processing contamination appears to be the cause of most cases of foodborne listeriosis (USFDA, 2003).

Dairy processing plants. As with other food processing plants, *L. monocytogenes* is regularly isolated from dairy processing and cheesemaking environments (Jacquet et al. 1993; Kabuki et al. 2004; Klausner and Donnelly, 1991; Makino et al. 2005; Pritchard et al. 1994, 1995; Sutherland and Porritt 1996; Unnerstad et al. 1996; Wagner et al. 1996). Although readily inactivated by typical pasteurization parameters, pasteurized milk and milk products have been implicated in outbreaks and

sporadic cases of listeriosis (Dalton et al. 1997; Fleming et al. 1985; Linnan et al. 1988; McClaughlin et al. 1990; Ryser 1999) as the result of post-processing contamination. Much like the dairy farm, dairy processing plants can contain highly diverse groups of *L. monocytogenes* strains including those of clinical significance (Arimi et al. 1997). Despite the variability between studies, dairy processing plants appear to be the least contaminated facilities when compared to those processing meat, poultry (Chasseignaux et al. 2001; Chasseignaux et al. 2002), or fish (Autio et al. 1999; Eklund et al. 1995; Hoffman et al. 2003; Lappi et al. 2004; Norton et al. 2001b; Thimothe et al. 2004; Wulff et al. 1996).

Contamination rates reported for *Listeria* spp. and *L. monocytogenes* in various types of dairy processing plants range from 7.7 to 76.2% and 7.7 to 30.2%, respectively (Charlton et al. 1990; Cox et al. 1989; Pritchard et al. 1994; Walker et al. 1991). However, rates as high as 100% have been reported for dairy processing plants with adjacent farms (Pritchard et al. 1994). In fact dairy processing facilities with a contiguous farm may be at higher risk of contamination than those facilities without an on-site dairy farm (Pritchard et al. 1994) where on farm sources of *Listeria* spp. such as dairy cattle, raw milk and silage can subsequently contaminate dairy processing environments (Arimi et al. 1997). Subtyping, more specifically ribotyping, has been used to illustrate the link between these farm sources of *L. monocytogenes* and dairy processing environments (Arimi et al. 1997). Although high isolation rates (57.9%) have been reported for on farm dairy processing facilities (Pritchard et al. 1994), the overall prevalence of *Listeria* spp. in farmstead dairy processing plants manufacturing cultured

dairy products, including cheese may be comparatively low (Ho et al. 2007) as substantially higher site isolation rates as high as 64.5% have been reported for other dairy processing facilities (Charlton et al. 1990; Flanders et al. 1995; Walker et al. 1991).

The risk of product contamination in cheese plants is thought to be greater due to increased exposure to the environment during manufacture, hooping, cutting and packaging compared to fluid milk production. Small scale production, by local producers, involving a large amount of hand contact could also contribute to a higher probability of contamination (Uhlich et al. 2006). However, in a study of cheese plants in California, only 9.8% (4 of 41) and 4.9% (2 of 41) were positive for *Listeria* spp. and *L. monocytogenes*, respectively (Charlton et al. 1990). In a study by Pritchard and others (1994) plants producing fluid milk, frozen milk products and dairy ingredients all had significantly higher rates of contamination than those producing cultured dairy foods such as cheese alone or in combination with fluid products.

The size and age of a cheese plant may also impact the incidence of listeriae. In a study of *Listeria* contamination within and between three Latin-style fresh cheese processing plants, the largest, most recently constructed plant showed the lowest frequency of contamination while the smallest establishment showed the highest frequency attributed to poor facility design and layout (Kabuki et al. 2004). Conversely, in another study a significantly higher recovery rate was reported for larger plants compared to small and medium sized attributed to heavier traffic, and longer operating schedules allowing for extended periods of wet conditions (Walker et al. 1991).

Of the six species of *Listeria*, *L. innocua*, followed by *L. monocytogenes*, appear to be the most frequent inhabitants of dairy processing environments (Cox et al. 1989; Pritchard et al. 1994) where single species appear to predominate not only within plants, but in given sites as well (Walker et al. 1991). Drains appear to be the most frequently contaminated site (Cox et al. 1989; Gabis et al. 1989; Ho et al. 2007; Kells and Gilmour, 2004; Walker R. L. 1991) and appear to be a useful in assessing the presence or absence of *Listeria* spp. in a general location of a plant (Charlton et al. 1990). Other commonly contaminated sites include floors, coolers and areas of pooled water (Cotton and White, 1992; Ho et al. 2007; Kells and Gilmour 2004; Walker et al. 1991). Recovery of *Listeria* spp. from surfaces in direct contact with water suggests that reducing the amount of pooled water aids in controlling pathogens in the dairy plant (Pritchard et al. 1995) as *L. monocytogenes* can survive in aerosols created by the use of high pressure water hoses during cleaning thus transferring cells from reservoirs such as drains and floors throughout the environment (Spurlock and Zattola, 1991). Employees can also serve as a source of *Listeria* contamination and dissemination, emphasizing the risks associated with rotating employees through multiple assigned duties and departments (Autio et al. 1999).

Raw materials as source of contamination. Results from environmental surveys conducted in other industries may also provide insight into the contamination patterns of *Listeria* spp. in processing plants. The processing of cold smoked fish is similar to that of many high risk cheeses in that there is no listericidal step involved in production. Furthermore, these products are kept refrigerated and consumed without further heating

(Autio et al. 1999). As with dairy processing plants, contaminated raw ingredients are believed to be the source of environmental *L. monocytogenes* contamination. Early surveys, however, demonstrate that raw ingredients rarely test positive and that contamination occurs further down the processing line at critical sources including brining and employee contact (Autio et al. 1999). Contamination of the fish processing environment appears to be due to strains already present in the plant environment (Hoffman et al. 2003) which in turn serve as the source of food product contamination (Autio et al. 1999; Norton et al. 2001b; Rorvik et al. 1995). Although contaminated raw materials can serve as a source of finished product contamination (Eklund et al. 1995), subtyping data suggest that not all types found in raw materials are seen in finished products (Norton et al. 2001b). Therefore the debate remains as to whether or not raw materials serve as a source of finished products contamination. While the presence of *Listeria* spp. notably *L. innocua*, in processing facilities suggests contamination with raw milk as documented during the FDA Dairy Safety Initiatives (Kozak et al. 1996), the low prevalence of *Listeria* spp. in raw milk receiving rooms indicates that the dairy farm and raw milk are not the primary sources of *Listeria* spp. in the plant (Charlton B. R. 1990). Additionally Walker and colleagues (1991) found no significant differences in contamination levels between plants receiving raw milk compared to those receiving pasteurized milk (Walker et al. 1991).

Control of Listeria spp. in the processing plant. The results of numerous environmental sampling surveys emphasize the need for sanitation programs aimed at environmental control of *Listeria* spp. notably *L. monocytogenes*. Additionally the

identification of contamination sources is necessary in order to design more effective control and monitoring programs in an effort to minimize the dissemination and subsequent contamination of food contact surfaces and finished products. Although plant sanitation and environmental control may not completely eliminate *Listeria*, it does decrease the level of contamination (Walker et al 1991; Wulff et al. 2006).

The presence of pathogens in food processing facilities can be effectively controlled when cleaning and disinfection programs are applied correctly to equipment and environments when hygienically designed and well maintained (Holah et al. 2002). The use of appropriate materials that are easily cleaned and sanitized in addition to sanitary design are important factors in reducing cross contamination (Kabuki et al. 2004). The replacement of old equipment and wooden shelves with those made of stainless steel in addition to improvements in disinfection systems has been shown to reduce *Listeria* spp. contamination levels in cheese plants (Menendez et al. 1997). Brine solutions should also be tested regularly as *L. monocytogenes* can survive over 200 days in commercial brine at 4°C (Larson et al. 1999). Although regulations may prohibit, the addition of sodium hypochlorite at levels of 10-100ppm can inhibit this survival (Larson et al. 1999).

In October of 2003 the USDA Food Safety and Inspection Service (USDA/FSIS) implemented a random, risks-based verification testing program for *L. monocytogenes* for RTE meats. While designed for meat, these programs can also apply to cheese products where establishments producing RTE products with no lethality treatment rely solely on sanitation measures. These plants are required to develop written programs such as

HACCP and SSOPs to control *L. monocytogenes* (9 CFR Part 430) and to verify program effectiveness through frequent environmental monitoring and end product testing. As a result the incidence of *L. monocytogenes* in RTE meat products has been reduced 70% since mandatory HACCP was implemented in 1998 (FSIS, 2003). The implementation of environmental monitoring has shown to reduce, if not eliminate, the presence of *Listeria* spp. in cheese processing facilities (Wagner et al. 1996). Comprehensive environmental monitoring programs incorporating molecular subtyping tools facilitate the detection of sporadic and persistent contamination (Kabuki et al. 2004). Such programs are necessary to monitor the effectiveness of sanitation and good manufacturing practices. Strict hygienic control at the point of production followed by improved temperature control after manufacture and shorter shelf lives may help prevent the contamination of soft cheeses from raw or pasteurized milk with *L. monocytogenes* and subsequent proliferation in cheese to levels of public health concern (McClaughlin et al. 1990).

Behavior in natural cheese.

Current regulations (21 CFR 133) permit the manufacture of numerous varieties of cheese from raw milk provided that these cheeses are aged for a minimum of 60 days commonly at temperatures ≥ 1.67 °C (35°F) to ensure safety. The ability of a pathogen to survive in a cheese is determined by a combination of parameters that act as hurdles to microbial growth including intrinsic qualities such as water activity (a_w), pH, acidity, and salt content. As a result many hard cheeses made from raw milk and aged for 60 days or

more have an excellent food safety record due to the interaction of these factors to render cheeses microbiologically safe (Donnelly, 2001).

The behavior of *L. monocytogenes* in natural cheese varieties is also a function of microbial injury and subsequent increases in the duration of lag phase. Generally speaking, lag time represents the period during which cells adjust to their new environment (Robinson et al. 1998). The duration of lag phase depends on the amount of biosynthetic and homeostatic processes that a cell needs to undergo to adapt to its environment and prepare for cell division and the rate it is able to do this work (Robinson et al. 1998). Increases in the lag time of *L. monocytogenes* can also result from microbial injury as previously reported for *L. monocytogenes* (Cheroute-Vialette et al. 1998; Dykes and Moorhead 2000; Guillier et al. 2005; Vasseur et al. 1999). In *L. monocytogenes*, NaCl concentrations above a critical level and pH values close to the limit for growth may result in cell damage and subsequent repair thus increasing the duration of lag phase (Robinson et al. 1998). As a result, the onset of pathogen growth in some cheeses is likely to be delayed due to microbial injury incurred from the presence of salt, and the low pH environment (Gay et al. 1996). Although temperature does not seem to have the same effect on lag time as salt or pH, lag increases rapidly at temperatures below 15°C at higher salt concentrations (Robinson et al. 1998).

It has also been shown that the duration of the lag phase of *L. monocytogenes* under stresses such as salt (Robinson et al. 2001) or conditions simulating soft cheese ripening (Gay et al. 1996) is also affected by inoculum size. It is possible that under very unfavorable growth conditions cells will die during the extended lag phase (Pascual et al.

2001). Given a very small inoculum size, there is the possibility that due to heterogeneity in cellular stress responses within a population, all the cells in the inoculum could die and no growth would occur (Pascual et al 2001).

In an effort to determine the fate of pathogens in cheese, numerous challenge studies have been conducted going back to the mid 1900s. Although pathogen population levels in milk are generally low as previously described, challenge studies typically employ initial levels approaching 4-5 log CFU/ml in order to observe subsequent changes that occur during processing. Regardless of initial inoculation, the physical entrapment of bacterial cells within the curd matrix results in a 6 to 10-fold increase in pathogen concentrations depending on cheese variety (Bachmann and Spahr, 1995; Buazzi et al. 1992; Mehta and Tatini, 1994; Ryser and Marth, 1987; Yousef and Marth, 1988; Yousef and Marth, 1990).

Semisoft and hard cheeses. U.S. standards (21 CFR 133) loosely define semisoft cheeses as those that contain more than 39 percent, but not more than 50 percent moisture, and their solids contain not less than 50 percent of milkfat. Similarly, hard cheeses are those that contain not more than 39 percent of moisture, and their solids contain not less than 50 percent of milkfat. Cheeses like Cheddar fall into the category of hard cheese by physical definition but differ due to its unique manufacturing process. As a result it is difficult to discuss the behavior of pathogenic bacteria, including *L. monocytogenes* in cheeses by name. Although specific standards exist, cheeses within a standard may vary considerably in terms of physicochemical composition.

Growth of *L. monocytogenes* during the manufacture of Colby (Yousef and Marth, 1988) and Cheddar including reduced fat and stirred curd varieties (Mehta and Tatini, 1994; Ryser and Marth, 1987) has been demonstrated. Although pathogen levels typically decline during aging of these cheeses, *L. monocytogenes* has been shown to survive well beyond the mandatory 60 day holding period. Duration of survival, however, is dependant on initial inoculation levels as well as moisture and salt contents. In contrast, the manufacturing process of Swiss cheese which includes the cooking of curds at 50°C to 53°C, results in pathogen inactivation (Buazzi et al. 1992). Further reductions occur during brining and the early stages of aging with complete inactivation following 60-80 days of ripening at 24°C (Buazzi et al. 1992). Similar studies on Swiss cheese varieties have demonstrated complete inactivation of *L. monocytogenes* within 24 hours of manufacture when manufactured from raw milk (Bachmann and Spahr, 1995). Hard grating cheeses such as Parmesan undergo similar cooking temperatures (~52°C) for even longer durations up to an hour, which in combination with small curd size, results in the expulsion of whey producing a dense and low-moisture cheese. These conditions, in addition to brining and aging, result in the rapid decrease in *L. monocytogenes* populations with complete inactivation occurring between 60 and 120 days depending on strain (Yousef and Marth, 1990). Additionally, large inocula of *L. monocytogenes* (4-5 log CFU per gram) introduced as post-processing contaminants were unable to survive on the surface and interior of freshly manufactured hard Italian-type cheeses (Ryser, 1999).

Soft cheese. As previously discussed, numerous outbreaks and sporadic cases of listeriosis have been linked to the consumption of soft cheeses (Bannister, 1987; De Buyser et al. 2001; Farber et al. 1990; Goulet et al. 1995; Linnan, et al. 1988; Lunden et al. 2004; Ryser, 1991) and in 2005, the FDA issued a health advisory warning consumers not to consume soft cheeses made from raw milk (USFDA, 2005). While this warning was more focused on illegally produced Hispanic-style cheeses, the lack of Standards of Identity for the various cheese varieties can create much confusion as soft cheeses, for which there is no standard, comprise a wide range of cheese types ranging from “Hispanic-style” types like queso fresco to surface ripened varieties such as Camembert each with varying risks associated. Specific standards of identity are critical to food safety as the characteristics of the specific cheese variety dictate the potential for growth and survival of microbial pathogens. Even the FDA in its risk assessment of RTE foods recognized that the risk associated with the consumption of different varieties of soft cheese including fresh, soft-ripened, and soft unripened cheese varies (USFDA, 2003). Furthermore different names can be used to describe the same cheese as Mexican-style cheeses such as queso fresco are similar in appearance, texture and taste. This causes problems from both a regulatory and epidemiological standpoint especially when large scale illegal importation of noncommercial cheeses from Latin America occurs (MacDonald et al. 2005).

The survival of *L. monocytogenes* in soft cheese has been demonstrated in the laboratory by numerous investigators. In cheeses acidified without the addition of starter culture such as queso blanco types (pH ~5.2 to 5.3), *L. monocytogenes* survives and in

some cases grows during aging depending on the acid used as an acidulant (Glass et al. 1995). *L. monocytogenes* has also been shown to survive longer than 90 days with only slight reductions in population levels in cheese with considerably low pH and high salt concentrations such as Feta (pH 4.6 to 5; Papageogiou and Marth, 1989). In other unripened varieties of soft cheeses such as cottage cheese, *L. monocytogenes* is eliminated through aggressive cooking (57.2°C for 30 min) and subsequent reductions in pH (from 6.65 to 4.7; El-Shenawy and Marth 1990; Ryser et al. 1985; Schaack and Marth, 1988). Surviving cells are also unlikely to grow during refrigerated storage when pH is below 5.5 (Schaack and Marth, 1988). .

The lack of a curd cooking step employed in the manufacture of surface-ripened cheeses such as Camembert and Brie-types combined with relatively high moisture allows for the survival of *L. monocytogenes* during manufacture. When *L. monocytogenes* (strains Scott A, OH, and CA) was inoculated in cheese milk used for Camembert-type cheese manufacture, counts in wedge and interior samples decreased 10-1000 fold during the first 17 days of ripening, an observation attributed to the low pH (<5.5) and storage temperature (15-16°C; Ryser and Marth, 1987). Morgan and colleagues (2001) noted a decrease in, but not complete elimination of *L. monocytogenes* populations during the manufacture and ripening of raw goat's milk soft-ripened cheese. From a milk inoculation level of 100 CFU/ml, the number of *L. monocytogenes* decreased until day 14 on the surface and day 28 in the interior where it remained close to 1.5 log CFU per gram. This observation was attributed to combined inhibitory effect of low pH and the activity of lactic starters. *L. monocytogenes* was only detectable through

dual enrichment in cheeses manufactured from milk contaminated with 10 CFU per milliliter. In a study by Gay and others (1996), *L. monocytogenes* Scott A populations declined and were unable to grow in Richard's broth adjusted to pH 4.8 used to simulate Camembert cheese at the beginning of ripening.

The heat treatment of the milk used for manufacture may also play a role in pathogen survival where, unlike pasteurized milk, raw milk may have a protective effect against pathogenic bacteria (Donnelly, 2001). Growth of pathogens such as *L. monocytogenes* can be inhibited by competitive flora including *Lactobacillus bulgaricus*, *Lactobacillus plantarum* and to a lesser extent by *Lactococcus lactis*, *Lactococcus cremoris* and *Streptococcus thermophilus*, likely due to competition for nutrients and a decrease in pH (Pitt et al. 2000). Gay and Amgar (2005) investigated the fate of *L. monocytogenes* during the manufacture and ripening of bloomy rind (soft-mold-ripened) cheese manufactured from either raw versus pasteurized milk and found that the lag phase and time to a 10^3 increase in *L. monocytogenes* levels, were twice as long in raw milk bloomy rind cheese compared to its pasteurized counterpart, likely due to the microbiological composition of raw milk, notably thermophilic lactobacilli and yeast. Conversely, Ramsaran and others (1998) reported that the growth of *L. monocytogenes* during the manufacture and ripening of Camembert was unaffected by the use of a nisin producing starter or raw milk when compared to the use of pasteurized milk. Some cheese varieties, including Camembert types utilize *Geotrichum candidum* (a yeast like fungus) to deacidify the cheese surface during ripening. A compound produced by *G. candidum*, D-3-phenyllactic acid, is active against both Gram-positive and Gram-

negative bacteria and has been shown to inhibit the growth of *L. monocytogenes* over a finite period of time (Dieuleveux et al. 1998a, 1998b).

In addition to survival during manufacture, mold-ripened cheeses are highly susceptible to surface contamination during ripening (Ryser, 1999). Post processing contamination of soft surface-ripened cheese is of particular concern as pathogen growth parallels the increasing pH during ripening (Ryser and Marth, 1987). The growth of *L. monocytogenes* on the surface of pasteurized milk Camembert-type cheese has been investigated using relatively high inoculation levels (~ 500 CFU/20cm², 2-4 log CFU/sample; Back et al. 1993; Genigeorgis et al., 1991; Ryser and Marth, 1987). However, the repeated sampling and experimental aging conditions, including the sealing of samples in plastic containers or foil, do not represent conditions commonly employed in commercial practice. In surface-ripened cheese, growth typically initiates at pH values between 5 and 6 (Ryser and Marth, 1987; Millet et al. 2006; Ramsaran et al. 1998). With an optimal pH for growth that is neutral or slightly alkaline (Farber et al. 1989), an increase in pH during ripening creates a favorable environment (in terms of pH) for pathogen growth (Ryser and Marth, 1987). If present, unlike other pathogens, *L. monocytogenes* can survive and continue to grow during refrigerated storage due to its psychrotrophic nature. In a study by Little and Knochel (1994), only the psychrotrophic pathogen *Yersinia enterocolitica* was able to grow when introduced as a post processing contaminant on stabilized Brie, whereas populations of both *Salmonella* and *Bacillus cereus* declined at 4 and 8°C. Ryser and Marth observed 2-3 log cycles of growth in three strains of *L. monocytogenes* surface inoculated onto 10 day old Camembert-type cheese

at 6°C reaching maximum population levels of approximately 3 to 5 log CFU/g with final pathogen level consistent with those found in contaminated soft-ripened cheeses at retail (Greenwood et al. 1991).

Given the results of scientific challenge studies it is not surprising that surface-ripened cheeses were some of the first from which listeriae were detected and to be implicated in outbreaks of listeriosis. Despite this knowledge, current regulations permit the manufacture and importation of semi-soft and soft ripened cheeses from raw milk provided that these cheeses are properly aged (60 days at ≥ 1.67 °C). Due to renewed interest in specialty cheeses, domestic artisan and farmstead producers are manufacturing semi-soft and soft cheeses from raw milk, using the 60 day holding standard to achieve safety.

While the incidence of pathogens in raw milk from commercial dairy farms is well documented, there is an absence of data in the literature regarding the microbiological quality and pathogen prevalence in raw milk for the manufacture of artisan and farmstead cheese, which are often made from raw milk. The first manuscript of this dissertation examined the overall quality and pathogen prevalence in noncommingled raw milk destined for farmstead cheese manufacture throughout the summer production season. Given that environmental contamination of food products with *L. monocytogenes* is an important factor to consider in risk assessments such as the Joint FDA/Health Canada Public Health Risk Assessment regarding *L. monocytogenes* in soft-ripened cheese currently underway and that limited data is available on the survival of *L. monocytogenes* introduced as a post processing contaminant on cheese, the second

study examined the growth potential of *L. monocytogenes* introduced as a post processing contaminant on surface-mold-ripened soft cheese as affected by two initial inoculation levels and the use of pasteurized or raw milk over time. Prevention of post-processing contamination centers on environmental control of *Listeria* spp. As with raw milk though, there is a lack of data regarding the incidence and ecology of *Listeria* spp. in artisan and farmstead cheese processing facilities. Thus the third manuscript examined the incidence and distribution of *Listeria* spp. in small scale farmstead cheese processing environments across multiple facilities over time to identify points of control for use in designing more effective control and monitoring programs. This study also compares the efficacy of three protocols for the detection and isolation of *Listeria* spp., including *L. monocytogenes*, from environmental samples.

The overall aim of this dissertation was to conduct research to fill data gaps in the scientific literature that consider the threat of emerging pathogens in the raw milk supply and farmstead cheese processing environment as well as the role of post processing contamination on the safety of aged raw milk cheese particularly at the farmstead level.

Chapter 1: Low incidence of foodborne pathogens of concern in raw milk utilized for farmstead cheese production

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Pathogen incidence in farmstead raw milk.

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Abstract

Overall milk quality and prevalence of four target pathogens in raw milk destined for farmstead cheesemaking was examined. Raw milk samples were collected weekly from June to September, 2006, from 11 farmstead cheese operations manufacturing raw milk cheese from cow (5), goat (4), and sheep (2) milks. Samples were screened for *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp., and *Escherichia coli* O157:H7 both quantitatively (direct plating) and qualitatively (PCR). Overall, 96.8% of samples had standard plate counts <100,000 CFU/ml, 42.7 % of which were <1000 CFU/ml.

While no federal standards exist for coliforms in raw milk, 61% of samples tested conformed to pasteurized milk standards under the Pasteurized Milk Ordinance (PMO) at <10 CFU/ml. All cows' and sheep's milk as well as 93.8% of goats' milk samples were within the limits of the PMO for somatic cell count (SCC). Of the 11 farms, 8 (73%) were positive for *S. aureus*, detected in 34.6% (46/133) of milk samples. *L. monocytogenes* was isolated from 3 milk samples (2.3%), two of which were from the same farm. *E. coli* O157:H7 was recovered from 1 sample of goat's milk for an overall incidence of 0.75%. *Salmonella* spp. were not recovered from any of the 133 samples.

The findings of this study demonstrate that most raw milk intended for farmstead cheesemaking is of high microbiological quality with a low incidence of pathogens.

These data will help inform risk assessments which evaluate the microbiological safety of farmstead cheeses, particularly those manufactured from raw milk.

U.S. Standards of Identity for cheese and cheese products (42) permit the manufacture of more than 30 varieties of cheese from raw milk, most of which must be aged for a minimum of 60 days at a temperature no less than 35°F (1.67°C). U.S. Federal regulations (43) governing milk, however, do not include microbiological standards for raw milk used for the manufacture of raw milk products, including the presence of pathogenic bacteria. Due to renewed interest in specialty cheeses, small-scale artisan and farmstead producers are manufacturing numerous varieties of cheese from raw milk, including those that present higher risk such as soft surface ripened cheeses.

Unpasteurized milk has been a known vehicle of foodborne disease since the inception of the dairy industry. In the early 1900's poor sanitation, improper milk handling and animal health issues resulted in numerous milkborne disease outbreaks (37). With the gradual implementation of pasteurization, the risk of milkborne infection was greatly reduced (2). Since the late 1970's, changes in agricultural practices, food processing operations and the globalization of the food supply has resulted in the emergence of newly recognized foodborne pathogens in the United States (2) including *Listeria monocytogenes*, multi-drug resistant *Salmonella* and *Escherichia coli* O157:H7. From 1973 to 1992, 46 raw milk associated foodborne disease outbreaks, resulting in 1,733 illnesses, were reported to the Centers for Disease Control (CDC) with *Campylobacter* and *Salmonella* constituting a majority of cases (16). As a result of these outbreaks, in 1987 a final rule was published requiring mandatory pasteurization of all milk and milk products intended for human consumption entering interstate commerce (21 CFR Section 1240.61(a)). Federally, the Grade "A" Pasteurized Milk Ordinance

(PMO) provides guidance to ensure that fluid milk is produced safely. Milk intended for cheesemaking, however, is subject to different regulations as determined by individual states and not necessarily governed by the PMO (47); where some states allow the use of Grade B, or manufacturing grade milk for the production of cheese (e.g. NY). Grade B milk is produced under less stringent hygienic guidelines and may be of higher risk for the presence of pathogens than that of Grade A (19).

The presence of pathogenic bacteria in raw milk is well documented (21, 24, 29, 30, 36, 44, 45) as the dairy farm itself can serve as a reservoir. Fecal contamination of teats, udder surfaces, and milking machines can result in subsequent contamination of milk (18). While the presence of pathogens in the farm environment does not always lead to the contamination of bulk tank milk, artisan cheeses produced in small processing facilities on dairy farms (farmstead) may be at an elevated risk for the presence pathogens due to the potential for environmental contamination of raw materials from the farm environment (32). The excessive workload of on-farm dairies may also deter producers from employing proper food hygiene practices (39). For these and other reasons, the use of raw milk for the production of farmstead cheese may be perceived as high risk. Typically a cheese classified as “farmstead” (e.g. The American Cheese Society at <http://www.cheesesociety.org>) is made with milk from the farmer’s own herd, or flock, on the farm where the animals are raised. The reduction in transport from using on-farm produced milk may help prevent increases in microbial populations that occur from additional surface contact and pumping during gradient transport (4). The reduced time from milking to cheesemaking also limits the outgrowth of undesirable bacteria that may

be present in the milk (47). Extended holding of cooled milk in the bulk tank allows for the selective growth of psychrotrophic pathogens including *L. monocytogenes* as well as spoilage flora, all of which are negative contributors to milk and cheese quality and safety (23).

While the incidence of pathogens in milk from commercial dairy farms is well documented, there is an absence of data in the literature regarding the microbiological quality and the prevalence of pathogenic bacteria in raw milk for the manufacture of artisan and farmstead cheeses (7), which are often made from raw milk. Data concerning changes in bacterial levels in milk from a given farm over time are also scant (7). The objective of this study was to fill data gaps in the scientific literature considering the threat of pathogens in raw milk intended for farmstead cheesemaking. We aimed to examine the overall quality and pathogen prevalence in raw milk destined for farmstead cheese manufacture throughout the main production season.

Materials and Methods

Milk collection. Study farms meeting the definition of farmstead were chosen based on geographic location, willingness to participate, and type of milk used for cheese manufacture (raw or pasteurized). While all study farms produced raw milk cheese, and thus was the focus of the study, some also produced additional products from pasteurized milk. The majority of farms included in this study produce milk and cheese seasonally. A total of 138 raw milk samples were collected weekly, on the same day each week, from 11 farmstead cheese operations located throughout the state of Vermont during the main production season between June and September 2006. Study farms manufactured raw

milk cheese from cow (5 farms, 67 samples), goat (4 farms, 49 samples), or sheep (2 farms, 22 samples) milk. The number of farms investigated represents approximately one third of Vermont's farmstead producers. Information regarding the species associated with individual study farms is not provided to ensure the confidentiality of participant identities. Herd sizes were generally small with cow, goat and sheep dairies ranging from approximately 18-100, 25-75, and 20-90 animals, respectively. In general, study farms kept animals on pasture for the duration of this study and were all milked using automated systems. Samples were aseptically collected by the farmer/cheesemaker from the farm bulk tank, milk cans or cheese vat following agitation and either placed in a refrigerator prior to pickup or immediately stored on ice for transport to the laboratory. Temperatures during transport were monitored using a multipurpose temperature data logger (SP125, Dickson, Addison, Ill.) to assure samples were not temperature abused. Due to the fact that milk was collected immediately prior to cheese manufacture, and in order to minimize bacterial population shifts during refrigeration (23), all samples were processed within 8 hours of collection.

Standard plate, coliform and somatic cell counts. Standard plate counts were determined using Petrifilm™ Aerobic Count Plates (3M™ Microbiology, St. Paul, Minn.) incubated $48\text{h} \pm 3\text{h}$ at $32^{\circ}\text{C} \pm 1^{\circ}\text{C}$ (AOAC® Official Method 986.33). Resulting counts were rounded to two significant figures at the time of conversion to standard plate count (SPC). Coliform counts were determined using Petrifilm™ Coliform Count Plates (3M™ Microbiology) incubated for $24\text{h} \pm 2\text{h}$ at $32^{\circ}\text{C} \pm 1^{\circ}\text{C}$ (AOAC® Official Method 986.33). All plates containing 25-250 CFU/ml were enumerated. Counts outside this

range are displayed in tables as estimates. For the determination of somatic cell counts (SCC), vials containing bronopol were filled upon arrival at the laboratory, shaken to ensure even distribution, and shipped to the Vermont Dairy Herd Improvement Association. Somatic cell counts were determined using a Bentley Somacount TM 500 (Bentley Instruments, Inc. Chaska, Minn.) calibrated to cow's milk.

Quantitative pathogen detection and isolation. Target pathogen population levels were determined through surface plating one milliliter of raw milk over 6 plates (167µl each) of CHROMagar™ (CHROMagar™ Microbiology, Paris, France) chromogenic agar media formulated for each specific target pathogen (CHROMagar™ Staph aureus, CHROMagar™ Salmonella, CHROMagar™ O157, and CHROMagar™ Listeria). CHROMagar™ Salmonella was modified through the addition of cefsulodin at 5 mg/liter (Sigma-Aldrich, St. Louis, Mo). CHROMagar™ O157 was modified to include potassium tellurite (Dynal, Invitrogen, Carlsbad, Calif.) at 2.5 mg/liter, cefixime (Dynal) at 0.025 mg/liter and cefsulodin (Sigma-Aldrich) at 5 mg/liter. All samples were plated in duplicate. Typical colonies, as defined by the manufacturer, were enumerated following 24h incubation at $37 \pm 0.5^{\circ}\text{C}$. All presumptive colonies enumerated were purified on tryptic soy agar (Becton Dickinson, Franklin Lakes, NJ) with 0.6% yeast extract (Becton Dickinson) added (TSAYE) and incubated for 24h at $35 \pm 0.5^{\circ}\text{C}$. A single colony from each TSAYE plate was then inoculated into 9ml tryptic soy broth (Becton Dickinson) with 0.6% yeast extract added (TSBYE) and incubated for 24h at $35 \pm 0.5^{\circ}\text{C}$ for confirmation by PCR as described below. Samples yielding at least one

confirmed colony for the target pathogen were deemed positive for the presence of that organism.

Qualitative pathogen detection and isolation. The Dupont™ Qualicon BAX® Q7 automated PCR system (BAX PCR; DuPont Qualicon, Wilmington, Del.) was employed for the qualitative detection of target pathogens according to the manufacturer's instructions. Each method has been validated by Health Canada, Health Products and Food Branch, Food Directorate, Bureau of Microbial Hazards published in the Compendium of Analytical Methods Volume 3 (available at: <http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3>). All PCR positive enrichments were streaked to respective CHROMagar™ media for isolation.

For the detection of *Salmonella* spp., 25ml of raw milk were added to 225ml of lactose broth and incubated for 24 ± 2 h at 35°C as recommended in the FDA Bacteriological Analytical Manual (BAM; Ch. 5. *Salmonella*. Available at: <http://www.cfsan.fda.gov/~ebam/bam-5.html>). Ten microliters of the enriched sample were then inoculated into 500µl of Brain Heart Infusion broth (BHI; Becton Dickinson) and incubated at 37°C for 3h. Five microliter aliquots of enriched BHI culture were removed and processed using the *Salmonella* spp. assay for BAX PCR according to the manufacturer's instructions.

Twenty-five milliliters of raw milk were enriched in 225ml of Complete Selective Enrichment Broth (CSEB) and incubated for 24 ± 2 h at 30°C for the detection of *L. monocytogenes*. One hundred microliters of the enriched sample were then transferred to 9.9 ml of MOPS-Buffered *Listeria* Enrichment Broth (MOPS-BLEB) for secondary

enrichment for 22 ± 2 h at 35°C . Aliquots (5 μl) of MOPS-BLEB were removed and processed using the *L. monocytogenes* assay for BAX PCR according to the manufacturer's instructions.

For the detection of *E. coli* O157:H7, 25ml of milk were enriched in flasks containing 225ml of Enterohemorrhagic *E. coli* (EHEC) Enrichment Broth (EEB) and incubated for 24h at $37 \pm 0.5^{\circ}\text{C}$ in an orbital shaker (Lab line Max -Q 2508. Barnstead International, Dubuque, Iowa.) as recommended by the FDA BAM (Chapter 4a, Diarrheagenic *Escherichia coli*. available at: <http://www.cfsan.fda.gov/~ebam/bam-4a.html>). The BAX PCR assay for *E. coli* O157:H7 was then run on 5 μl aliquots of enrichment cultures according to the manufacturer's instructions.

S. aureus was detected solely through direct plating of raw milk on CHROMagar™ Staph (CHROMagar™) as previously described. Two samples originally outside our predicted range (Table 4) were reanalyzed the following day (<24h at 4°C). Isolates obtained were purified on TSAYE incubated for 24h at $35 \pm 0.5^{\circ}\text{C}$. Purified colonies were Gram stained, examined for catalase activity and regrown in BHI incubated for 24h at $35 \pm 0.5^{\circ}\text{C}$. To test for coagulase, 10 μl of BHI enrichment were added to 0.5 ml of reconstituted coagulase plasma with EDTA (BBL™ Coagulase Plasma, Becton Dickinson), mixed thoroughly and incubated at 35°C . Tubes were examined periodically over 6h periods, up to 24h. Any degree of clotting within 24h was considered a positive test. Randomly, coagulase positive isolates were ribotyped for *S. aureus* confirmation as described below.

Ribotyping: Isolates of *L. monocytogenes* and *S. aureus* were subtyped through ribotyping using the automated Riboprinter® Microbial Characterization System (DuPont Qualicon) according to the manufacturer's instructions using the restriction endonuclease *EcoRI* (Qualicon).

Statistical analyses. The resulting data were analyzed using the SPSS for Windows (version 15.0.1, SPSS Inc., Chicago, Ill.). Cell counts reported in tables as <1 CFU/ml represent actual observed average colony counts of 0 or 0.5 CFU/ml. Actual observed average colony counts were utilized for statistical analyses. Additionally, estimated colony counts were not included in analyses. The general linear models procedure (GLM) was used to perform univariate analyses of variance (ANOVA) on log transformed SPC and CC data to detect differences in cell counts within and between species over time. CC data were not log transformed for analyses due to the presence of numerous null values. Spearman's rank correlation coefficient was utilized to determine correlations between cell counts and pathogen presence. Results with values $p < 0.05$ were considered significant. Histograms were constructed using Microsoft Excel (Microsoft, Seattle Wash.).

Results

Standard Plate and Coliform Counts. A total of 122 and 123 samples from 11 farms were analyzed for standard plate (SPC) and coliform counts (CC), respectively. One study farm's SPC and CC data were removed from analyses after well water contamination was discovered through routine state testing as per the PMO, which in turn contaminated the dairy. An additional four SPC values were removed from analysis due

to the addition of starter culture prior to milk collection (farm K). Changes in SPCs on individual farms over time are presented in Table 1. SPCs of the remaining 118 samples ranged from 10 to 9,300,000 CFU/ml with mean, geometric mean (geo-mean) and median values of 99,000, 1,400, and 1,450 CFU/ml, respectively (Table 1). The geometric mean is a log-transformation of data obtained over a period of time which tends to dampen the effects of outlying data. The European Union (E.U.) employs this method to take account of seasonal variations as regulatory samples are taken multiple times over a two or three month period. Geometric means may better represent the average value in this study due to the outlying data, which may bias the mean. There was no significant effect of sampling week or month on SPC levels observed on individual farms or all farms as a whole. Overall, 96.5% of samples analyzed had SPCs <100,000 CFU/ml with 79.6% <10,000 CFU/ml. Notably, 43.2% were <1000 CFU/ml with the remaining 16.9% below 100 CFU/ml (Figure 1). When analyzed separately by species (Figure 2), cow's milk SPCs were all below 100,000 CFU/ml with 82.4% <10,000 CFU/ml. Over half (54.4%) of cow's milk SPCs were below 1000 CFU/ml with almost one quarter (24.6%) below 100 CFU/ml. Mean, geo-mean and median SPC values were 4900, 680 and 700 CFU/ml, respectively. Although goats were the only species producing milk with SPCs >100,000 (8.2%; Figure 2), 73.4% were below 10,000 CFU/ml, 24.5% less than <1,000 CFU/ml and 12.2% under 100 CFU/ml. Mean, geo-mean and median values for goat's milk SPCs were 230,000, 2,543 and 3,100 CFU/ml, respectively. While no samples of sheep's milk had SPCs less than 100 CFU/ml, all were below 100,000 CFU/ml with mean, geo-mean and median SPCs of 6,900, 2,758 and

1,750 CFU/ml, respectively. The overwhelming majority of sheep's milk samples (75%) were below 10,000 CFU/ml with 16.7% containing less than 1,000 CFU/ml. Observed differences in SPCs between species were significant ($p=0.029$) with lower SPC values observed in cows milk samples.

A total of 121 milk samples were analyzed for coliform bacteria. Coliform counts observed in all milks ranged from <1 to 9,600 CFU/ml with mean, geo-mean and median values of 263, <1 , and 5.5 CFU/ml, respectively. Changes in CCs on individual farms over time are presented in Table 2. There was no significant effect of sampling week or month on coliform levels observed on individual farms or all farms as a whole. Out of 121 samples analyzed for coliforms, 84.3% contained less than 100 CFU/ml with 61.2% <10 CFU/ml (Figure 3). Of the remainder, 11.6% contained CCs between 101-1000 CFU/ml with the remaining 4.1% between 1001-10,000 CFU/ml. When separated by species (Figure 4), 89% of cow's milk samples were <100 CFU/ml with 62% below 10 CFU/ml. Mean and median CCs for cow's milk alone were 343 and 6.5 CFU/ml, respectively. Seventy-six percent of goat's milk had CCs less than 100 CFU/ml with 55% below 10 CFU/ml. For sheep's milk samples, 92% were under 100 CFU/ml with 75% under 10 CFU/ml. Goat and sheep's milk samples mean and median CC values were 212 and 4.5 CFU/ml, and 24.6 and 2.8 CFU/ml, respectively. No significant difference ($p=0.723$) in CCs between species was observed even with outliers removed ($p=0.265$), likely due to variance within and across species. Although correlations between SPC and CC were limited, a significant positive correlation was observed on Farm E ($r = 0.629$, $p<0.05$).

Somatic cells counts. SCCs (Table 3) differed significantly between species ($p < 0.001$) with goat's milk containing the highest levels, followed by sheep and then cow's milk. Additionally, since SCC standards vary by species, statistical analyses were conducted separately. Figure 5 shows the relative frequency distribution of SCCs for cow, goat and sheep's milk. All cow's milk samples ($n=61$) contained $< 750,000$ somatic cells/ml with 83% under 400,000 cells/ml. The remaining 17% fell between 400,000 and 700,000 cells/ml with milk mean, geo-mean and median values of 179,147, 127,270 and 123,000 cells/ml, respectively. Three goat's milk samples exceeded 1,000,000 cells/ml with 81% below 700,000 cells/ml and almost half (46%) under 400,000 cells/ml. Goat's milk SCCs were 512,292 cells/ml on average with a median value of 400,000 cells/ml. All sheep milk samples contained less than 700,000 cells/ml. Mean SCC for raw sheep milk was 383,955 cells/ml with a median value of 460,000 cells/ml. Significant correlations between SCC and SPC on farm G ($r = 0.767$, $p < 0.01$) and CC on farm F ($r = 0.615$, $p < 0.05$) were observed. SCCs were not however correlated to the presence of pathogens, including *S. aureus*.

Pathogen incidence. Of the 11 farms, 8 (73%) had at least one milk sample test positive for *S. aureus* over the course of the study including samples from all species. Coagulase positive isolates formed grade 4+ clots within 4-6 hours of incubation in all but one case which formed a similar clot following 18 hours of incubation. All selected isolates of coagulase positive staphylococci were identified as *S. aureus* via ribotyping. As the most common pathogen detected, *S. aureus* was isolated by direct plating in 33.3% (44/132) of samples at an average level of 250 CFU/ml (median: < 1 CFU/ml) as

shown in Table 4. Seventeen of 62 (27.4%) cow's milk samples, 9 of 49 (18.4%) goat's milk samples and 18 of 21 (85.7%) ewe's milk samples were positive for *S. aureus*.

L. monocytogenes was isolated from 2.3% (3/133) of all milk samples or 4.8% (3/62) of cow's milk samples. The three isolates of *L. monocytogenes* were obtained from 2 of the 5 cow farms. Two of the three isolates were detected by direct plating at <1 CFU/ml from the same farm, 10 weeks apart. Subtyping revealed that the two isolates were of different ribotypes (DUP-1030B and DUP-1045B). The first isolate obtained in sampling week 1 was of the same ribotype (DUP-1030B) as the isolate recovered from a milk sample from the other positive farm through enrichment in week 6. Detection following enrichment also suggests contamination levels <1 CFU/ml. *E. coli* O157:H7 was recovered from one sample (0.75%) of caprine milk following enrichment. Study farms associated with pathogen presence in raw milk samples are not provided to ensure the confidentiality of participants. *Salmonella* spp. were not recovered from any of the 133 samples (0%) analyzed. Subsequent examination of raw milk cheeses produced from contaminated milks following manufacture and at time of sale using the BAX PCR system as previously described, failed to detect or recover viable pathogens (data not shown). In another instance the contaminated raw milk was pasteurized shortly after sample collection.

Discussion

This study examined the trends in bacterial and somatic cell loads in raw milk for the production of farmstead cheese through the repeated sampling of farms over time. While laboratory procedures were performed in a central laboratory and remained

consistent throughout the study, the methodology and experimental design differ from many of the studies presented in this discussion which typically consisted of single samplings over a large number of farms of a single species. Because of this, direct comparisons are not intended and associations should be interpreted with caution.

Results of sampling conducted early in the study revealed widespread contamination of one study farm's dairy, which was later traced to well water. These findings highlight the benefits of routine raw milk and water screening in farmstead cheese operations for microbiological quality. While there are no standards for SPC in raw milk for the manufacture of raw milk products in the U.S., the SPC limit for pre-pasteurized raw milk is 100,000 CFU/ml (43); only 3.5% of all samples exceeded this limit. Furthermore, 79.6% of milk samples contained less than 10,000 CFU/ml, the target recommended as best practice for raw milk intended for the manufacture of raw milk cheese. Overall, cow's milk SPCs were significantly lower than both sheep and goats. When analyzed separately by species, 100%, 91.8%, and 100% of cow, goat and sheep's milk samples contained SPCs <100,000 CFU/ml, respectively (Figure 2). All cow's milk dairies also had geometric mean SPCs well below 100,000 CFU/ml (Table 1) as per E.U. regulations for raw cows' milk (10). From a regulatory stand point and in terms of range, cow's milk (as well as goat and sheep) SPCs observed in this study are similar to those obtained as part of the National Animal Health Monitoring System (NAHMS) Dairy 2002 survey (44). In the NAHMS survey, 92.1% of cow's milk samples were within the PMO limits compared to 100% (96.5% all species combined) reported here. The differences lie in overall distribution, where 54.4% of cow's milk (43.2% all species

combined) samples in our study had SPCs <1000 CFU/ml compared to only 12% in the NAHMS survey where the majority of SPCs fell between 1,000 and 4,999 (48%) compared to only 21% (27.1% all species) in our study. Of 855 raw milk samples from New York State taken over 2 years, only 26.8% of samples contained SPC <5000 with the remaining 73.2% at >5000 CFU/ml (*1*), almost twice the percentage reported in the NAHMS survey (40%; *44*) and almost three times that observed in our study (25%). The geometric mean of cow's milk SPCs reported here (680 CFU/ml) is also substantially lower than that observed in New York State (11,400 CFU/ml; *1*). Mean SPCs for cow's milk observed in the present study (4,900 CFU/ml) were also far lower than the 26,600 CFU/ml calculated using data from approximately 90% of the commodity fluid milk produced in Vermont during the same sampling period (Vermont Agency of Agriculture 2006, unpublished data). While all sheep's milk samples were within the standards of the PMO for raw milk to be pasteurized (100,000 CFU/ml), 8.2% of goat's milk exceeded the standards. All sheep and goat milk farms maintained geometric mean SPCs (Table 1) within the E.U. limits for raw milk intended for the manufacture of raw milk products by a process that does not involve any heat treatment (<500,000 CFU/ml; *10*). Additionally, 73% and 75% of goat and sheep milk samples, respectively, were below the target set for best practice when making raw cow's milk cheese at <10,000 CFU/ml with only two goat's milk samples exceeding that recommended for small ruminants at <250,000 CFU/ml. It has been suggested that since SPC between sheep and goats do not differ significantly, similar microbiological limits can be used for both species (27). This is true of our study when considering geometric mean and median values, but not arithmetic

means due to the presence of outlying data. Median SPC for goat and sheep bulk tank milk observed in our study of 3.5 log CFU/ml (goat) and 3.44 log CFU/ml (sheep) are more than 1 log lower than levels typically reported in the literature including reports from Switzerland of 4.69 log CFU/ml and 4.78 log CFU/ml for goat's and sheep's milk, respectively (27). Goat's milk mean SPC observed in the present study (5.36 log CFU/ml) are higher than those reported for raw goat's milk destined for the manufacture of raw milk cheese in Italy (mean 4.7 log CFU/ml; 13) as well as a grade A research herd in Oklahoma (4.16 CFU/ml; 47) as the likely result of outlying data. Although data on coliform levels in goat's milk is limited, our results (2.33 log CFU/ml) are in agreement with others (13, 47).

While no U.S. federal standards exist for coliform levels in raw milk, 61.2% of all samples tested (n =121) conformed to PMO standards for Grade "A" pasteurized (not raw) milk at <10 CFU/ml (Figure 3) while 84.3% were within the recommended standards for raw milk for raw milk cheese at <100 CFU/ml. Levels of coliform bacteria reported in the literature vary widely. Ranges observed here for all milks (<1 CFU/ml to 4.89 log CFU/ml) are similar to those reported by Desmaures and others (8) for cow's milk from the Camembert region of Normandy (<1 to 4.34 log CFU/ml) and Jayarao and Wang (18) in the U.S. (0 to 4.7 log CFU/ml). When analyzed separately, the maximum coliform count in cow's milk was one log less than all milks combined with a range from <1 CFU/ml to 3.98 CFU/ml. In a study by Desmaures and others (8), 84% of cow's milk samples contained coliform levels <2 log CFU/ml, similar to the 90.3% in our study yet slightly higher than found for dairy herds in the U.S. at 73% (18) and 76.5% (1) as

well as in Belgium at 67.1% (6). Mean coliform counts (2.29 log CFU/ml) were also similar to those reported in Belgium (2.45 log CFU/ml; 6), yet ten-fold lower than the U.S. (3.4 log CFU/ml; 18).

All cow's milk samples (n=65) were within the limits of the PMO for SCC at <750,000 cells/ml. The overall geometric mean SCCs for all cow's milk samples of 127,270 cells/ml was within the E.U. limit of 400,000 cells/ml. On an individual basis, only one farm exceeded this limit. Average SCC in farmstead cow's milk reported here (179,147 cells/ml) was nearly half that of producers shipping milk in the state of Vermont during the same sampling time frame (341,608 cells/ml; Vermont Agency of Agriculture 2006, unpublished data). Compared to average SCC from throughout the year, our results are still below the national average of 288,000 cells/ml and the state of VT average (250,000 cells/ml) for milk from Dairy Herd Improvement herds during 2006 (26) as well as the recommended level in raw milk for raw milk cheese (<250,000 cells/ml). All sheep's milk samples (n=22) were within the limits of the PMO at <750,000 cells/ml. Although average SCC in raw goat's milk was the highest of the species in the present study (512,000 cells/ml), it was slightly lower than that reported for goat's milk used for raw milk cheese production in Italy (13). Three (6.2%) samples exceeded the limits for SCC in goat's milk under the PMO; 93.8% of samples were in compliance. It is important to note that the instrument utilized to determine somatic cell counts for small ruminant milk samples was calibrated to cow's milk which may have affected the resulting counts reported here.

In addition to quality indicators, this study determined the prevalence of four target pathogens in raw milk for farmstead cheesemaking. Numerous studies have examined the prevalence of foodborne pathogens in bulk tank milk (19, 24, 29, 30, 36, 44, 45). The data however, varies greatly due to differences in geographic location, season, hygiene, herd size (36) and detection methodology.

As a result of the repeated sampling employed in our study, it is important to consider that resulting pathogen incidence rates reported here are likely higher than those that would result from single point-in-time surveys (20). In our study, 50 of 137 (36.5%) samples contained one or more pathogens. Overall pathogen prevalence rates reported in the U.S. range from 10.5%-32% (19, 20, 36), although target pathogens vary. Many surveys do not include *S. aureus* as a pathogen of interest, whereas *S. aureus* comprised 92% of the pathogens isolated in our study. If removed and only *Salmonella* spp., *E. coli* O157:H7 and *L. monocytogenes* are considered, the incidence of pathogenic bacteria drops drastically to 3%.

As a major causative agent of mastitis, *S. aureus* is one of the most common contagious pathogens infecting dairy cows. Commonly isolated from raw milk, *S. aureus* has been reported as the frequent pathogen associated with foodborne outbreaks related to cheese from raw or unspecified milk in France (5). *S. aureus* was detected in 17 of 62 (27.4%) cows' milk samples in our study, similar to the 25.1% observed by De Reu and others (6) in Belgium. Similar contamination levels were also observed in a study of *S. aureus* prevalence on a Norwegian dairy producing raw cow's milk cheese at 240 CFU/ml (21). Small ruminant milk can contain *S. aureus* as well, detected in 30-40% of

goat (27, 41) and ewe's (27) milk samples, including those destined for raw milk cheese manufacture (41). While the incidence in goat's milk observed here falls below this range, sheep's milk shows a higher incidence due to the small number of samples, most of which were taken from a single farm (n=12). The overall variation in *S. aureus* levels and isolation across and within species is likely due to the tendency for intermittent shedding of the organism. The lack of a positive correlation between SCC and *S. aureus* could be explained by the presence of other mastitis pathogens not included in the survey. Mean levels of *S. aureus* observed in this study (250 CFU/ml) are below the target for raw milk cheese manufacture of 500 CFU/ml and do not necessarily present a food safety risk as virulence lies in the production of heat stable enterotoxins, which generally occurs when populations exceed >5 log CFU/ml. Thus cheeses made from raw milk under E.U. regulation (11) must contain <5 log CFU/ml at the time during manufacture when the number of staphylococci is expected to be highest. If values >5 log CFU/ml are detected, the cheese batch must be tested for the presence of enterotoxins. *S. aureus* however is generally regarded as a poor competitor resulting in limited growth during cheesemaking in the presence of active starter culture. In order to better understand the risk of foodborne illness attributed to the presence of *S. aureus* on study farms, an examination of the genetic diversity and antibiotic resistance within our isolates as well as their propensity for toxin production warrants further investigation.

Dairy cattle are a known reservoir of shiga-toxin producing *E. coli*, including serotype O157:H7 (46). Incidence appears to be low (0.75%) in the bulk tank milk of cull cows (30) but may be higher (4.3%) in the raw milk obtained from case farms (46).

While potentially pathogenic, shiga toxin producing *E. coli* have been isolated from 2.4-3.8% of milk samples in South Dakota, western Minnesota (19) and Pennsylvania (20); none of the isolates were of serotype O157:H7. Similarly, no O157:H7 were detected in surveys conducted in southeast Scotland (3), Italy (25) or The Netherlands (17) in 500, 100 and 1011 samples, respectively. Although our sampling was conducted during late spring and summer when isolation rates are generally higher (30), *E. coli* O157:H7 was not detected in any cow's milk samples. Results are also not likely to have been affected by intermittent shedding as in other studies due to the repeated sampling of individual farms. Our data and that of others suggest that contamination of milk may be uncommon (3). Non-O157:H7 shiga toxin producing *E. coli* have also been detected in both goat and sheep milk at 16.3% and 12.7%, respectively (27). *E. coli* O157:H7 was however isolated from one (0.75%) sample of goat's milk through enrichment, suggesting a contamination level <1 CFU/ml. Contamination of goat's milk may not be uncommon as raw goat's milk was implicated in an outbreak of *E. coli* O157:H7 in British Columbia in 2001 (33), and was isolated from 1.7% of raw goat's milk samples in an Italian survey at 1.5 CFU/ml (13).

Salmonella spp. can be also found in the intestinal tracts of dairy cows which can serve as a major reservoir for human foodborne salmonellosis via fecal contamination and unpasteurized milk and milk products have been implicated in numerous outbreaks of salmonellosis (9). While low incidence rates from 0.1% to 2.9% have been reported in Europe (8, 34) and 0.17% in Canada (40), higher isolation rates from 1.5% to 8.9% of cow's milk have been reported in the U.S. (19, 20, 29, 36, 44) depending on geographic

location. In this study however, *Salmonella* spp. was not detected in any samples analyzed, similar to that observed in raw cow's milk and raw cow's milk cheese in Belgium (6). Although incidence data for small ruminant milk is limited, *Salmonella* spp. has been detected in goat's milk (13). Traditional culture methods for the isolation of *Salmonella* are time consuming and labor intensive and the use of PCR can improve detection in bulk tank milk (22) through enhanced sensitivity in a much shorter time. The failure to detect *Salmonella* spp. here is most likely due to low levels of the organism in milk, an issue possibly confounded by the enrichment protocol employed prior to PCR detection. Raw milk containing a high load of background flora, notably coliforms, may out-compete *Salmonella* spp. present at low levels during primary enrichment in lactose broth. Optimization of enrichment protocols for PCR is an area requiring further investigation.

Although the ruminant farm environment may serve as a significant reservoir for *L. monocytogenes*, including subtypes linked to human listeriosis (31), *L. monocytogenes* was detected in 3 samples of cow's milk at <1 CFU/ml for corresponding prevalence rates of 4.8% (3/62) for cow's milk and 2.3% (3/133) for all milks combined. Two isolates were recovered from the milk of a single farm, and although *L. monocytogenes* subtypes have been shown to persist in a herd's bulk milk (12, 28) our subtyping data suggests recontamination of milk from a separate source as opposed to a persistent contaminant. One of these two subtypes shared the same ribotype as a third isolate obtained from a separate farm six weeks later. Although genetically indistinguishable isolates were shared between processors, transmission is improbable due to the distance

and the lack of an epidemiological link between facilities. In accordance with our data, detection through direct plating and enrichment has shown that contamination levels are typically low ranging from <1-37 CFU/ml (12, 14, 24, 44) including milk of high microbiological quality for cheesemaking (7). While some surveys have failed to detect any *L. monocytogenes* in bulk tank milk (24), the incidence rate for cow's milk reported here is on the lower end reported in surveys conducted throughout the U.S. ranging from 2.8 to 7% (19, 20, 24, 28, 36, 44). Our results are also in accordance with detection rates from outside the U.S. ranging from 1 to 5.3% throughout Europe (14, 15, 34, 38, 45) and Ontario, Canada (40). Higher incidence rates up to 33.3% (15) have been reported for dairy silo milk likely due to the longer storage time and the commingling of milk from numerous producers (45). Although both goats and sheep are known carriers of *L. monocytogenes* (35), no samples of raw milk from small ruminant farms tested positive. Other investigators have reported prevalence rates of 2.56% in raw goat's milk (14) and 2.19% in sheep's milk (35) in Spain. In the latter study, failure to isolate from direct plating indicates contamination levels were less than 5 CFU/ml (35). *L. monocytogenes* prevalence reported here may however underestimate the yearly incidence as *Listeria* spp. contamination seems to increase in the winter months while animals are indoors, grouped together and feeding on silage (38).

The findings of this study indicate that most raw milk intended for farmstead cheesemaking was of high microbiological quality in reference to current U.S. and E.U. standards as well as in comparison to reports from the state of Vermont, the U.S. and abroad. Pathogen incidence and milk quality data is also similar to those reported in

surveys examining the quality of small scale and farmstead milk destined for the manufacture of raw milk products, including cheese, in European countries. This suggests that factors inherent to small scale operations such as the lack of milk pooling and holding, pasture feeding and very small herd sizes may play a role in the overall quality of raw milk. Milk quality however, may not correlate with milk safety. In accordance with others (4, 40, 44), our results suggest that one microbiological measurement, such as SCC or SPC (44), may not serve as a predictor of other bacterial values including pathogen presence and that compliance with bacteriological limits does not guarantee the absence of pathogenic bacteria.

It is apparent in the variation from farm to farm, regardless of species, that some operations practice strict hygienic controls and that additional effort is needed on others. While it has been suggested repeated sampling results in higher incidence of pathogen detection at the farm level (20), the incidence reported here is low in comparison to other surveys and therefore may be conservative. Our data suggest that if present, pathogen population levels are extremely low. However, improper storage of raw milk and raw milk products facilitates pathogen growth to levels of public health concern. Additionally, the physicochemical parameters of some cheeses produced from raw milk permit the survival and possible growth of certain pathogens. Since this study was limited to a small number of farms sampled over time, future investigations will include the sampling of farmstead operations representing multiple regions over each season. Further investigation is also warranted to determine sources of contamination in an effort to establish control programs. Therefore these data form part of an exposure assessment

and risk reduction plan to promote continuous production of microbiologically safe artisan and farmstead cheese. These data will also help inform risk assessments which evaluate the microbiological safety of farmstead cheeses, particularly those manufactured from raw milk.

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Figure Legends.

Figure 1: Relative frequency of raw milk standard plate counts (SPC) of all species (n=118).

Figure 2: Relative frequency of raw milk standard plate (SPC) counts in cow (■, n=57), sheep (□, n=12), and goat's (▣, n=49) milk.

Figure 3: Relative frequency of raw milk coliform counts in milk of all species (n=121).

Figure 4: Relative frequency of raw milk coliform counts (CC) counts in cow (■, n=63), sheep (□, n=12), and goat's (▣, n=47) milk.

Figure 5: Relative frequency of raw milk somatic cell counts (SCC) for cow (■, n=61), sheep (□, n=22), and goat's (▣, n=48) milk.

Tables

Table 1: Raw milk standard plate counts (CFU/ml) over time (weeks) by individual farmstead operation (A-K) ^c.

Week	Farm									
	A	B	C	D	E	F	G	I	J	K
1	900	41000	85	820	14000	16000	1200	410	30	^b
2	1700	76000	130	1200	1600	9300000	1800	79000	23	15000
3	3700	560	160	3700	1600	76000	7500	10	30	^b
4	7200	5500	150	17000	1300	150000	710	100	70	^b
5	1400	270	130	6900	1100	20000	1100	1700	110	^b
6	2800	530	200	3100	9300	20000	130	600	540	17000
7	1100	7400	120	4100	3300	1300000	650	100	37	^a
8	880	^a	93	1500	4500	1500	330	75	20	15000
9	3200	140000	170	1700	5700	9400	1700	130	120	22000
10	52000	3900	73	3300	^a	7300	18000	130	73	9600
11	^a	600	46	3700	^a	15000	230	89	380	13000
12	7800	4000	78	^a	1500	51000	950	450	19	^a
13	2500	1400	54	1800	11000	^a	700	110	25	16000
GM ^d	3000	4000	100	2900	3400	45000	1200	260	59	15000

^a: Not tested

^b: Data not present due to presence of starter culture

^c: Results are expressed as CFU/ml for ease of comparison with previous studies. ^d: Geometric mean

Table 2: Raw milk coliform counts (CFU/ml) over time (weeks) by individual farmstead operation (A-K).^b

Week	Farm									
	A	B	C	D	E	F	G	I	J	K
1	3	est.>500	<1	9.5	15	4.5	4.5	1	<1	6.5
2	<1	525	<1	34.5	52	est.>100	7	9600	<1	21.5
3	12	<1	<1	70.5	38.5	265	5.5	3	<1	17.5
4	23	<1	3.5	31	7.5	335	6.5	1.5	<1	1300
5	1.5	1	<1	38	4	1380	24	2.5	<1	13
6	5	<1	1	8.5	44.5	41	7	25	76	41.5
7	5.5	<1	<1	220	420	655	<1	4	<1	4
8	<1	^a	<1	17	110.5	85.5	5.5	<1	<1	58.5
9	2.5	4250	<1	23.5	420	450	7	8.5	<1	17
10	240	3.5	<1	19.5	^a	480	3.5	<1	<1	12
11	^a	<1	<1	3.5	^a	665	15	1	<1	8650
12	2	2	<1	^a	6	320	3	<1	<1	^a
13	<1	<1	3	17.5	425	^a	6.5	<1	<1	62

^a: Not tested

^b: Results are expressed as CFU/ml for ease of comparison with previous studies.

Table 3: Somatic cell counts (cells/ml) over time (weeks) by individual farmstead operation (A-K).

Week	Farm										
	A	B	C	D	E	F	G	H	I	J	K
1	400000	348000	264000	1213000	81000	264000	246000	566000	81000	62000	606000
2	606000	696000	230000	650000	50000	200000	200000	27000	66000	50000	460000
3	264000	857000	303000	^a	29000	373000	230000	^a	76000	71000	348000
4	606000	857000	283000	81000	33000	400000	187000	162000	123000	47000	325000
5	650000	919000	303000	303000	71000	650000	162000	93000	87000	62000	373000
6	47000	303000	152000	1493000	38000	123000	123000	650000	283000	41000	373000
7	460000	696000	246000	606000	54000	460000	141000	528000	200000	50000	325000
8	460000	^a	264000	492000	54000	492000	200000	214000	100000	29000	400000
9	492000	650000	325000	303000	115000	400000	230000	303000	141000	50000	460000
10	460000	606000	460000	460000	^a	985000	528000	107000	230000	71000	606000
11	^a	528000	348000	528000	^a	528000	187000	174000	162000	66000	400000
12	650000	919000	303000	^a	71000	1393000	214000	^a	132000	71000	^a
13	528000	800000	230000	303000	66000	^a	152000	^a	^a	66000	373000
GM ^b	404995	645895	276977	459340	55832	431190	201075	198587	126671	54972	411707

^a: Not tested

^b: Geometric mea

Table 4: *Staphylococcus aureus* counts on positive farms over time.

Week	Farm							
	A	B	D	F	G	H	J	K
1	53	<1	<1	1	<1	12500 ^b	<1	<1
2	18	<1	7	<1	28.5	15	<1	17
3	<1	<1	<1	<1	<1	^a	1.5	<1
4	8	<1	44	<1	39	72	7.5	<1
5	3	<1	4	<1	18.5	4.5	<1	<1
6	13	47.5	<1	<1	12.5	4	<1	25.5
7	55.5	51	<1	<1	19.5	8.5	<1	<1
8	5	^a	<1	<1	24	85.5	<1	<1
9	<1	56	<1	<1	26	59	<1	<1
10	9	11	<1	<1	20600 ^b	<1	2.5	<1
11	^a	<1	<1	<1	30.5	8.5	3.5	<1
12	^a	<1	^a	<1	26.5	^a	<1	^a
13	4	<1	<1	^a	24.5	^a	<1	<1

^a: Not tested

^b: Result represent counts following 24h holding at 4°C

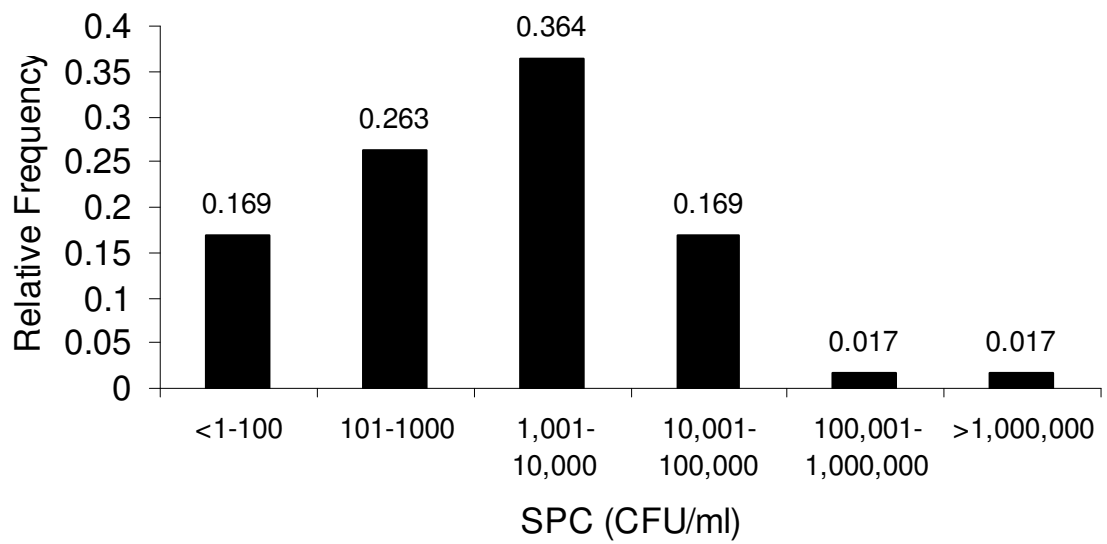


Figure 1: Relative frequency of raw milk standard plate counts (SPC) of all species (n=118).

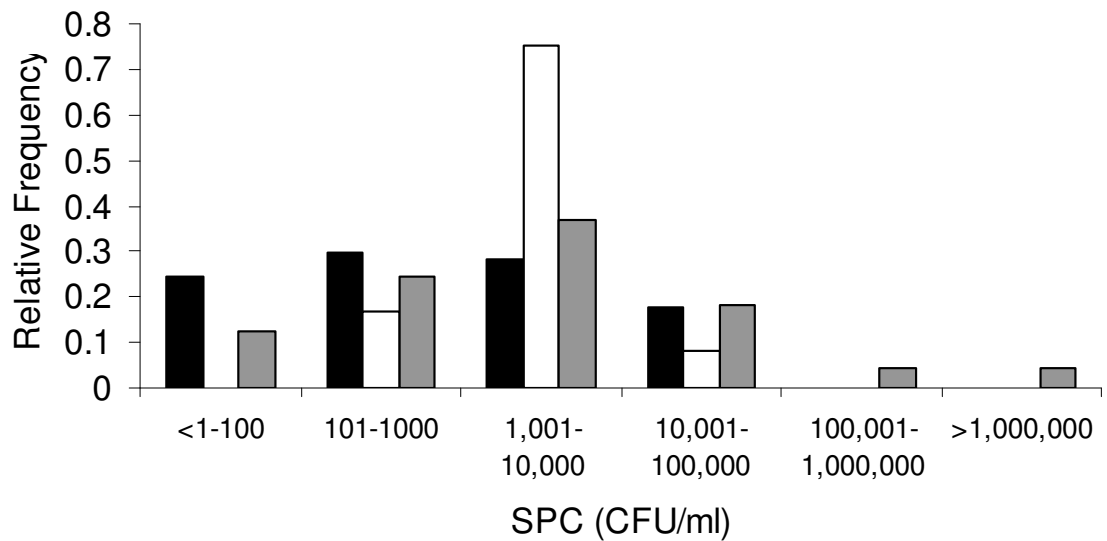


Figure 2: Relative frequency of raw milk standard plate (SPC) counts in cow (■, n=57), sheep (□, n=12), and goat's (▒, n=49) milk.

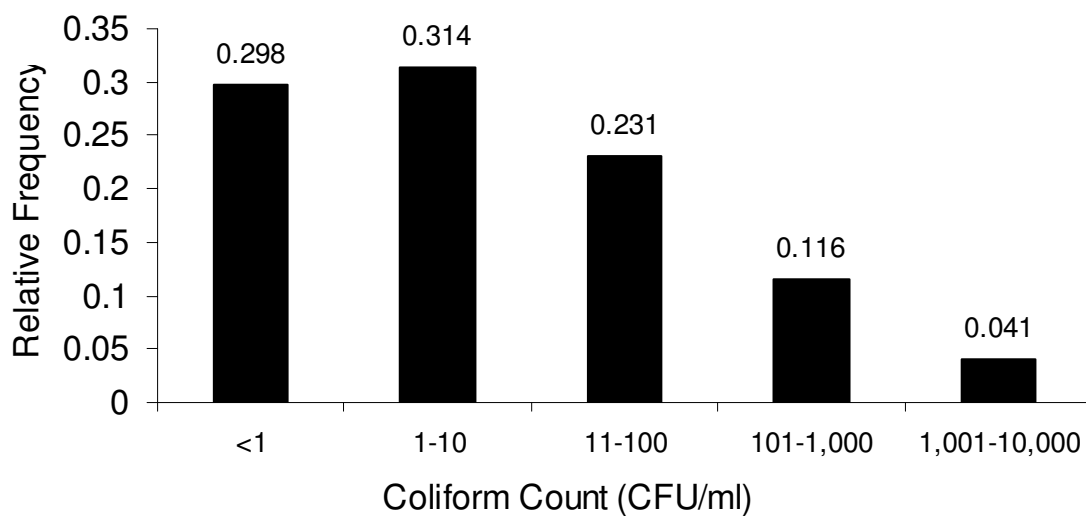


Figure 3: Relative frequency of raw milk coliform counts in milk of all species (n=121).

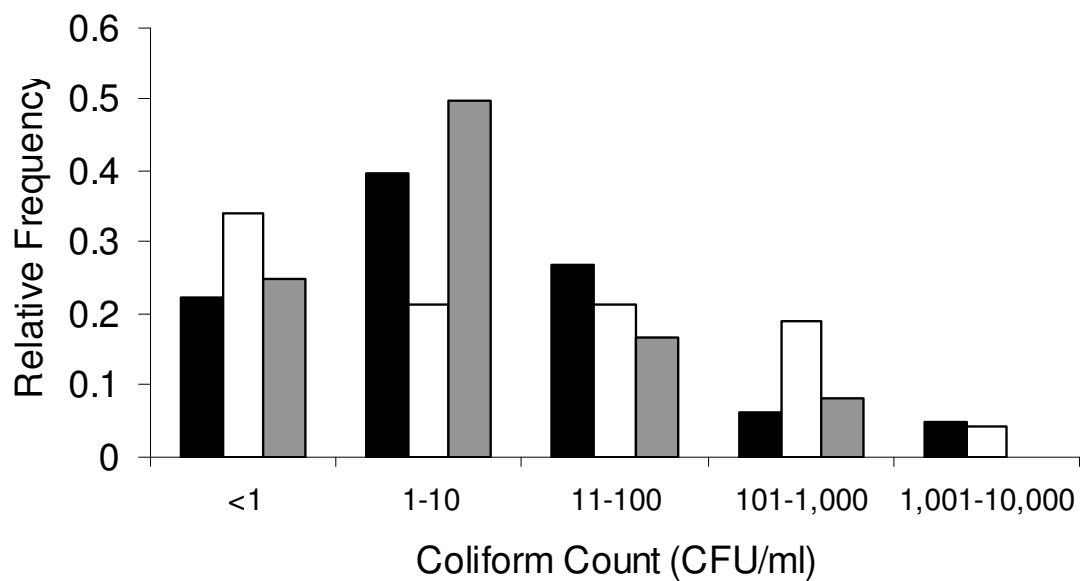


Figure 4: Relative frequency of raw milk coliform counts (CC) counts in cow (■, n=63), sheep (□, n=12), and goat's (■, n=47) milk.

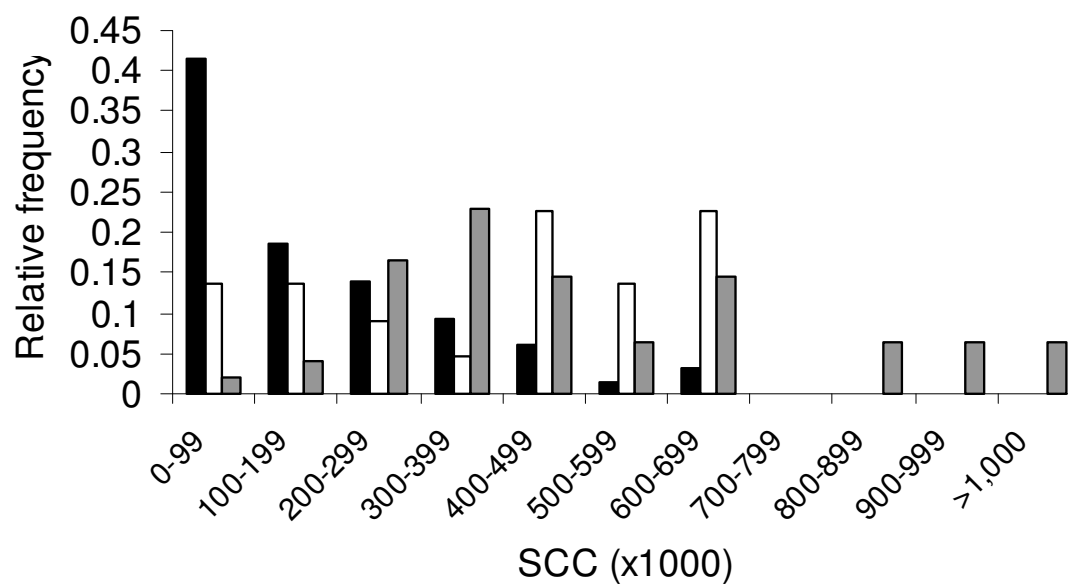


Figure 5: Relative frequency of raw milk somatic cell counts (SCC) for cow (■, n=61), sheep (□, n=22), and goat's (■, n=48) milk.

Chapter 2: The 60 day aging requirement does not ensure safety of soft surface mold ripened cheeses manufactured from raw or pasteurized milk when *Listeria monocytogenes* are introduced as post-processing contaminants.

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Survival of *Listeria monocytogenes* on soft surface mold ripened cheese.

Keywords: Keywords: *Listeria monocytogenes*, raw milk, pasteurized, cheese, soft surface mold ripened, recontamination.

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Abstract

Due to renewed interest in specialty cheeses, artisan and farmstead producers are manufacturing soft surface mold ripened cheeses from raw milk, using the 60 day holding standard (21 CFR 133.182) to achieve safety. This study compared the growth potential of *Listeria monocytogenes* on cheeses manufactured from raw or pasteurized milk and held for >60 days at 4°C. Final cheeses were within Federal Standards of Identity for soft ripened cheese, with low moisture targets to facilitate the holding period. Wheels were surface inoculated with a five-strain cocktail of *L. monocytogenes* to contain approximately 0.2 CFU/cm² (low level) or 2 CFU/cm² (high level), ripened, wrapped and held at 4°C. *Listeria* growth was initiated at day 28 for all treatments after initial population declines. From the low inoculation level, populations reached maximum levels of 2.96 ± 2.79 and 2.33 ± 2.10 log CFU/g after 60 days of holding, in raw and pasteurized milk cheese, respectively. Similar growth was observed in cheese inoculated at high levels where populations reached 4.55 ± 4.33 and 5.29 ± 5.11 log CFU/g for raw and pasteurized milk cheese, respectively. No significant differences ($p < 0.05$) were observed in pH development, growth rate, or population levels between milk types. Cheeses held for 60 days supported growth from very low levels of *L. monocytogenes* introduced as post process contaminants independent of the milk type. The safety of cheeses within this category must be achieved through control strategies other than aging and thus revisions of current federal regulations are warranted.

Introduction

According to the Centers for Disease Control and Prevention, *Listeria monocytogenes* had the second highest case fatality rate (20%) and the highest hospitalization rate (92.2%) of all foodborne pathogens (29). Numerous outbreaks and sporadic cases of listeriosis have been linked to the consumption of soft fresh cheeses (2, 5, 10, 24, 28), as well as soft surface ripened cheeses (16, 38). A subcategory of soft cheese, soft surface ripened cheeses are those which undergo further ripening through the external growth of yeast, mold and/or bacteria. Soft surface mold ripened cheeses include the well known Brie- and Camembert-types. According to a recent risk assessment (45), the risk associated with the consumption of fresh soft and soft ripened cheeses (moderate risk) differs from that of unripened soft cheese (high risk). In 2005, the U.S. Food and Drug Administration (FDA) issued a health advisory warning consumers not to consume soft cheeses made from raw milk (42). While this warning was more focused on illegally produced Mexican-style cheeses, the lack of Standards of Identity for the various cheese varieties can create much confusion as soft cheeses comprise a wide range of cheese types ranging from Hispanic or Mexican-style cheese such as soft unripened Queso Fresco, to surface ripened varieties such as Camembert-types, each with varying risks associated. The concern with soft surface mold ripened cheese is not necessarily the survival of *L. monocytogenes*, but the growth potential during aging and holding following increases in pH. Specific standards of identity are critical to food safety as the characteristics of the specific cheese variety dictate the potential for growth and survival of microbial pathogens. Many hard cheeses made from raw milk and aged for 60 days or more have an excellent food safety record due to the

interaction of factors including pH, salt content, and water activity (a_w) to render cheeses microbiologically safe (6). Current regulations (43) permit the manufacture of soft ripened cheeses from raw milk provided that these cheeses are aged for 60 days at $\geq 1.67^\circ\text{C}$ (35°F) to ensure safety. Raw milk cheeses that have not been properly aged are illegal in the U.S. and cannot be imported. Due to renewed interest in specialty cheeses, domestic artisan and farmstead producers are manufacturing soft surface mold ripened cheeses from raw milk, using the 60 day holding standard to achieve safety. In Europe, Protected Denomination of Origin (PDO) cheeses such as Camembert de Normandie, are required to be manufactured from raw milk in order to receive PDO status. Safety is not attained through aging (although aged a minimum 21 days), but through regulations specified in European Union (EU) Directives (N^o2073/2005) which establish microbiological criteria dependent upon whether cheese is made from raw versus thermized milk (9).

While raw milk may contain *L. monocytogenes*, the overall prevalence is low, with sporadic contamination and seasonal variability. When present, levels in bulk tank milk are typically below 3 CFU/ml (27, 30). Furthermore, *L. monocytogenes* is primarily an environmental pathogen, and the primary risk to cheese safety is that of post pasteurization environmental contamination from the cheese making/aging environment (19). Pasteurized or otherwise processed milk (4) and cheeses (8, 17, 19, 36) are each at risk of containing *L. monocytogenes* due to post processing contamination that can occur during manufacture, as well as ripening and washing (19). Recontamination of foods with pathogenic bacteria may be a frequent and important cause of foodborne outbreaks where post processing contamination is believed to be the cause of major outbreaks of

listeriosis attributed to the consumption of soft cheese (12, 17). Post processing contamination of soft surface ripened cheese is of particular concern as pathogen growth parallels the increasing pH during ripening (37). With an optimal pH for growth that is neutral or slightly alkaline, the increase in pH during ripening creates a favorable environment (in terms of pH) for pathogen growth (37).

If present, unlike other pathogens, *L. monocytogenes* can survive and continue to grow during refrigerated storage due to its psychrotrophic nature. The growth of *L. monocytogenes* on the surface of pasteurized milk Camembert-type cheese has been previously investigated using relatively high inoculation levels (~ 500 CFU/20cm², 2-4 log CFU/sample; 1, 15, 37). Additionally, the repeated sampling and experimental aging conditions, including the sealing of samples in plastic containers or foil, do not represent conditions commonly employed in commercial practice. From a risk assessment perspective, realistic inoculation levels and conditions are important as initial concentrations and atmospheric conditions affect the behavior of pathogens, including *L. monocytogenes*, in cheese (13, 25, 32). Furthermore, unlike pasteurized milk, raw milk may have a protective effect against pathogenic bacteria (6). Gay and Amgar (13) investigated the fate of *L. monocytogenes* added to milk during the manufacture and ripening of Camembert cheese manufactured from either raw versus pasteurized milk. It was found that the lag phase and time to a 10^3 increase in *L. monocytogenes* levels were twice as long in raw milk Camembert cheese compared to its pasteurized counterpart, likely due to the microbiological composition of raw milk, notably thermophilic *Lactobacillus* and yeast.

While *L. monocytogenes* has been studied extensively in both food and processing environments, and extensive risk assessments are available, limited data is available on the survival of *L. monocytogenes* introduced as a post processing contaminant on soft surface mold ripened cheese. Environmental contamination of food products is an important factor to consider in future assessments such as the Joint FDA/Health Canada Public Health Risk Assessment regarding *L. monocytogenes* in soft ripened cheese, which is currently underway. The present study examines the growth potential of *L. monocytogenes* introduced as post processing contaminants on soft surface mold ripened cheese as affected by (i) two initial inoculation levels and (ii) the use of pasteurized versus raw milk (iii) over the course of 70 days.

Materials and Methods

Cultures. *L. monocytogenes* strains used in this study (FSL R2-499, FSL N3-022 and FSL J1-119) were kindly provided by Dr. Martin Wiedmann at Cornell University (11). Additional strains utilized (F5069 and F5027), were obtained from the culture collection of Dr. Catherine Donnelly at the University of Vermont. Frozen (-78°C) stock cultures were inoculated into 9 ml of trypticase soy broth with 0.6% yeast extract added (TSBYE) and incubated at $30 \pm 1^\circ\text{C}$ for 18 h for 2 subsequent transfers prior to use. Viable numbers of *L. monocytogenes* in suspension for each culture were determined by aerobic plate counts after serial dilution on PetrifilmTM AC films (3M Microbiology, St. Paul MN) incubated at $32^\circ \pm 1^\circ\text{C}$ for 48 ± 2 h. Based on consecutive plate counts, equal proportions of cells from each culture were combined as a cocktail yielding $\sim 3 \times 10^9$ cells/ml. Freeze dried starter cultures including *Lactococcus lactis* subsp. *lactis*, *Lc. lactis*

subsp. *cremoris*, *Lc. lactis* subsp. *lactis* biovar *diacetylactis*, *Streptococcus salivarius* subsp. *thermophilus* and ripening cultures *Kluyveromyces lactis*, *Geotrichum candidum* and *Penicillium candidum* (EZAL MA011/MA019 and MD99/TO50/KL71/GEO17/SAM3; Danisco, Copenhagen, Denmark) were stored at -35°C until use.

Milk collection. Approximately 100 liters each of raw and pasteurized (non-homogenized) milks were obtained from a local dairy plant in 10 gallon sanitized stainless steel milk cans. Raw milk samples were collected before entering the pasteurizer whereas pasteurized milk samples were HTST pasteurized (72°C, 15 seconds) and collected bypassing the homogenizer. Mean temperatures of pasteurized and raw milks during transport (~90 min) were $4.83 \pm 0.70^{\circ}\text{C}$ and $6.76 \pm 0.26^{\circ}\text{C}$, with maximum values of $6.89 \pm 0.87^{\circ}\text{C}$ and $7.78 \pm 0.87^{\circ}\text{C}$, respectively. Milk cans were refrigerated ($3 \pm 1^{\circ}\text{C}$) immediately upon arrival until use the next morning ($22 \pm 2\text{h}$). *L. monocytogenes* was not detected in either raw or pasteurized milk through selective enrichment in *Listeria* Enrichment Broth (LEB) at 30°C for 24h followed by plating of 0.1ml on CHROMagar™ *Listeria* (CHROMagar Paris, France) incubated at 37°C for 24h.

Cheese manufacture. Soft surface mold ripened cheese was manufactured from 100 liters each of raw and pasteurized milk in separate vats on three different days as three independent trials. Each trial yielded ~35 cheeses of each milk type for a total of ~70 cheeses. With three trials, a total of ~210 cheeses were manufactured in this study, 120 of which were included in microbiological analyses. A 45 min delay between raw and pasteurized milk cheese manufacture was employed to allow enough time between

steps to avoid overlap, as well as time for thorough cleaning and sanitizing of utensils. Cheeses were manufactured with a slightly lower moisture content to endure the 60 day holding period, extending the ripening so that cheeses were in a consumable state at day 60. Temperature and acidification profiles were monitored and recorded using a temperature and pH data logger (Model DO 9505. Delta Ohm. Padua, Italy). Milk samples for compositional and microbiological analysis were taken upon filling of the vat, chilled and refrigerated (3°C) until use within 24h. For cheese manufacture, refrigerated raw and pasteurized milks (pH 6.7-6.8; TA 0.16-0.17%) were added to separate sanitized pilot scale double-O style, steam jacketed cheese vats (Kusel Equipment Co., Watertown, WI) in the University of Vermont pilot plant. Rehydrated Glucono delta-lactone (GDL; 30g/100 liters; Jungbunzlauer S.A., Marckolsheim, France) and CaCl₂ (10ml/100 liters) were added upon filling vats. GDL is used extensively throughout the E.U. as an acidulant in the manufacture of soft surface mold ripened cheeses and was therefore added in the manufacture of our experimental cheese to produce a food matrix of typical chemical composition. In the US, GDL, is a generally recognized as safe (GRAS; 21 CFR 184.1318) additive, and approved for use as a “pH control agent” as defined in 21 CFR 170.3(o)(23) in some foods. The action of GDL helps gradually decrease the pH during the lag period of the freeze dried culture providing consistent and controlled acidity development prior to the addition of rennet. Milk temperature was raised to between 36-39°C followed by the addition of lactic starter and ripening cultures (Starter: 2.25 Danisco Culture Units (DCU) MAO11/MAO19; 0.25 DCU MD99; 1.5 DCU TAO54/TAO50. Ripening: 0.3 DCU KL71; 0.2 DCU GEO17; 0.6 DCU SAM3). This temperature was maintained for 30-60 min until target set pH of 6.5-

6.55 was reached. Once proper pH was attained, calf rennet (20ml/100 liters, strength 1:15,000; New England Cheesemaking Supply, Ashfield MA) was added to the vat and milk was stirred for approximately 45 seconds. Cutting time was determined by multiplying the time of flocculation by 3. Once desired firmness was reached, the coagulum was cut into 2x2cm curds using sanitized stainless steel curd knives. Curds were then allowed to settle for 5 min, stirred for 5 min, allowed to rest for 5 min, and stirred for another 5 min. After an additional 5 min rest, 30% of the whey was removed. The curds were then gently stirred and transferred to plastic hoops (Model: M5-3864. Fromagex, Rimouski (Québec) Canada) to drain and turned regularly at 1.5, 3 and 8h after hooping. The initial draining temperature of 27°C was slowly decreased to 21°C at the time of dehooping. The following morning, once target pH of 4.8-4.9 was reached, cheeses were removed from hoops and placed in saturated brine (NaCl 270g/liter, pH 4.9, 12-13°C) for 70 min.

Drying, aging, and holding conditions. Upon removal from the brine, cheeses were transferred to racks sanitized with 70% isopropyl alcohol for the drying phase. Drying and ripening were conducted in a lab scale aging chamber constructed using a modified wine refrigerator (Model WC491BG, Avanti. Miami, FL). A small ultrasonic misting humidifier (Sunbeam Model 697, Jarden Consumer Solutions. Boca Raton, FL) provided moisture by atomizing deionized water into very fine cool mist particles that are easily absorbed in the air without affecting temperature. Humidity levels were controlled using a repeat cycle time switch (Talento 121, Grasslin Controls Co. Mahwah, New Jersey) set to predetermined on/off cycles to maintain desired humidity. For the drying

phase, the aging chamber was maintained at 14-15°C (57-59°F) and 80-85% relative humidity for 24h followed by the ripening phase at 13-14°C (55-57°F) and 90-95 % relative humidity for 12 ± 1 days. Once proper surface mold growth was achieved, cheeses were wrapped in 225x225mm multiplex wrapping paper (PVCF-23, White Mould. Fromagex. Rimouski (Quebec), Canada) and transferred to a standard refrigerator at $4 \pm 1^\circ\text{C}$ until day 70.

Bacterial inoculation. Cocktails of *L. monocytogenes* were serially diluted to obtain either low (10-20 CFU/ml) or high (100-200 CFU/ml) inocula. After the drying phase, cheeses were removed and one surface (100 cm²) was inoculated with 1 ml of the appropriate inoculum (high or low) using a sterile L-spreader to achieve low and high contamination levels of 0.1-0.2 CFU/cm² (low) and 1-2 CFU/cm² (high), respectively. Resulting cheeses from each trial (n = 40) were assigned to one of four treatments. Treatments consisted of raw milk, low inoculum (RL, n=10), raw milk, high inoculum (RH, n=10), pasteurized milk, low inoculum (PL, n=10) or pasteurized milk, high inoculum (PH, n=10). Inoculated cheeses were allowed to dry in a laminar flow hood (~10 min). The process was then repeated for the remaining opposite surface. Inoculated cheeses were then placed in the lab scale aging chamber as previously described for ripening.

Bacterial enumeration. The following samples were analyzed: milk, cheese after brining and drying step (19 hr), after blooming began (day 4) and during ripening on days 7 and 14. Samples were then taken during holding at 4°C on days 21, 28, 35, 42, 49, 56, 63 and 70. Milk standard plate counts (SPC) for both raw and pasteurized milks

were determined using Petrifilm™ Aerobic Count Plates (3M™ Microbiology, St. Paul, MN) incubated $48\text{h} \pm 3\text{h}$ at $32^\circ\text{C} \pm 1^\circ\text{C}$ (AOAC® Official Method 986.33). Resulting counts were rounded off to two significant figures at the time of conversion to SPC. Cheeses were analyzed out to day 70 to reflect growth during time in distribution and retail. Top and bottom surfaces of each cheese were analyzed separately in order to increase the sensitivity of detection. Results were then combined for analysis. For example, a cheese was considered positive if either a top or bottom sample was positive, whereas if both surfaces were negative, the cheese was considered negative. Whole top and bottom 100cm^2 surfaces (first 1-1.5cm, 100g) of each cheese were removed using a sterile cheese cutter and placed in separate sterile Whirlpak® bags (Nasco, Fort Atkinson, Wisconsin), appropriately diluted in sterile Butterfields phosphate buffer (BPB) and stomached for 3 min in a Stomacher® 400 circulator (Seward Limited, West Sussex, UK.). The resulting homogenate was serially diluted (10^{-1}) in BPB to facilitate enumeration. To reach a detection limit of ≥ 5 CFU/g, large (150 x 15mm) Petri dishes (25384-326 VWR) containing 30 ml of CHROMagar™ *Listeria* (CHROMagar, Paris, France.) were inoculated with 1ml of homogenate in duplicate. Refrigerated plates were allowed to dry in a laminar flow hood (~10 min) prior to inoculation. Following incubation at 37°C for 24 h turquoise colonies surrounded by a white halo were enumerated. Sugar tubes were utilized to further discriminate *L. monocytogenes* from *L. ivanovii*. Randomly, presumptive *L. monocytogenes* colonies were confirmed using automated PCR (BAX DuPont Qualicon, Wilmington, DE).

Physicochemical analysis of cheese. Physiochemical analyses were conducted on the milk before cheese manufacture, after dehooping and after the drying step. The following analyses were performed in duplicate: Protein (Kjeldahl method), pH (Accumet research AR150 with a flat tip electrode (Accumet Reference 13-620-46) Fisher Scientific International Inc. Hampton, NH), total solids (TS) (after drying to constant weight at 102°C), and chloride (Chloride Analyzer 926, Nelson Jameson, Marshfield, WI). Salt in moisture phase (SMP) moisture non-fat substance (MNFS) and fat in dry matter (FDM) were determined using the formulas:

$$\text{SMP} = (\text{salt} / (100 - \text{total solids})) * 100$$

$$\text{MNFS} = (100 - \text{TS} / (100 - \text{fat})) * 100$$

$$\text{FDM} = (\text{fat} / \text{TS}) * 100$$

Titrateable acidity (0.1 M NaOH, phenolphthalein indicator) was determined for the milk prior to and throughout manufacture. Ripening surface and cheese interior pH measurements were also taken at each microbiological sampling interval.

Statistical analyses. The resulting data were analyzed using the SPSS for Windows (version 15.0.1; SPSS Inc., Chicago, IL). The general linear models procedure (GLM) was used to perform univariate analyses of variance (ANOVA) to determine the effect of milk type on the rate of change in mean log CFU/g, as well as the effect of time on changes in both pH and mean log CFU/g. T-tests were utilized to compare SPC, cheese composition as well as temperature and humidity during drying, aging and holding between raw and pasteurized milk and cheeses. Non-parametric comparisons of mean log counts of *L. monocytogenes* on individual days were made using Mann-

Whitney tests. Results with P values < 0.05 were considered significant. Data are expressed as mean \pm standard error of the mean (SEM) of the three independent trials.

Results

Milk. SPC between raw and pasteurized milk were significantly different ($p < 0.001$), with mean counts of 6900 and 20 CFU/ml, respectively. *L. monocytogenes* was not detectable in any raw or pasteurized milk samples prior to cheese manufacture.

Cheese composition. According to U.S. Federal Standards of Identity (43), soft ripened cheeses produced from raw milk must be held for at least 60 days at no more than 35°F (1.67°C) to ensure safety. In order to produce a 60 day old cheese with a stabilized paste typically found in a younger soft surface mold ripened cheese, cheeses were manufactured with a slightly lower moisture target at dehooping. The Federal Standards of Identity also specify that soft ripened cheese must contain at least 50% fat in dry matter (FDM). Despite decreased moisture, all trials, raw and pasteurized, contained >50% FDM with an average of 52% (SD \pm 0.01) for raw and 52% (SD \pm 0.02) for pasteurized. Overall, no significant differences in general composition were observed between milk types (Table 1).

Drying, aging, and holding conditions. Raw and pasteurized cheeses were placed in separate chambers for drying and aging and therefore subject to variations in conditions. However, no significant differences were observed in average temperature or average relative humidity between milk types.

Changes in pH. In all trials, target pH at dehooping of 4.80-4.85 was achieved. Figures 1 and 2 demonstrate the changes in pH observed on the surface and in the interior

of cheeses, respectively, over time at each microbiological sampling interval. Univariate analysis of variance reveals that the linear change in interior pH as well as surface pH over time was not significantly different between two milk types at both inoculation levels (Interior high level: $p=0.373$, low level: $p=0.586$; Surface high level: $p=0.423$, low level: $p=0.480$). Differences in inoculation level did not significantly affect the rate of pH development ($p=0.691$). However, there was a very significant overall change in pH over time ($p<0.001$) at both inoculation levels.

Survival of *L. monocytogenes* during drying, aging, and holding. Following inoculation, *L. monocytogenes* was undetectable in all samples tested and remained below the detection limit (≥ 5 CFU/g) until growth initiated at day 21 (RL) or 28 (remaining treatments) with corresponding mean pH values of 5.16 (RL), 5.76 (PL) (Figure 3a), 5.63 (RH) and 5.91 (PH) (Figure 3b). In some cases at the low inoculation level, *L. monocytogenes* remained undetectable throughout aging and holding even at 70 days of age as shown in Table 2. Due to the very low level of inoculation and the subsequent undetectable nature of cells during the initial 3-4 weeks, only data from the initiation of growth to day 70 was used in the univariate analyses of variance. Log values of mean counts were used for analysis due to the presence of zeros (null values) in the data. Figure 4a shows the growth of *L. monocytogenes* over time on both raw and pasteurized milk cheese at the low inoculation level. The linear change in log mean CFU/g over time from the onset of detectable growth was not significantly different between the two milk types ($p=0.206$). Furthermore mean counts by individual days did not differ significantly between milk types ($p<0.05$) except for day 42 where mean counts of *L. monocytogenes* were moderately higher in the pasteurized milk cheese ($p=0.1$).

Figure 4b shows the growth of *L. monocytogenes* over time on both raw and pasteurized milk cheese at the high inoculation level. The linear change in log mean CFU/g over time from the onset of detectable growth was again not significantly different between the two milk types ($p=0.962$). Mean counts by individual days did not differ significantly between milk types ($p<0.05$). For both treatments there was a very significant change in log mean CFU/g over time ($p<0.001$). From the low inoculation level, populations reached maximum levels of 2.96 ± 2.79 and 2.33 ± 2.10 log CFU/g after 60 days of holding, in raw and pasteurized milk cheese, respectively. More extensive growth was observed in high level inoculated cheese where population levels reached 4.55 ± 4.33 and 5.29 ± 5.11 log CFU/g after 60 days of holding, for raw and pasteurized milk cheese, respectively. Populations of *L. monocytogenes* grew at significantly higher rates from the high inoculation level compared to the low ($p=0.032$) despite similar changes in surface and interior pH. This seems to suggest population growth potential is dependent on initial contamination levels. However, if we eliminate cheeses negative for *L. monocytogenes* and only analyze the positive counts (Figure 4), there is no significant effect of initial inoculation level on the rate of growth ($p=0.096$) although there is still no difference in rate of growth between milk types ($p=0.963$).

Discussion

While no standards exist in the U.S. for compositional characteristics of soft ripened cheese beyond FDM, the Codex Alimentarius (21) classifies our experimental cheese as a semi-soft, full fat, surface mould ripened cheese. Additionally the Codex Alimentarius International Individual Standard for Camembert (22) states that with a

minimum FDM between 45-55%, moisture must not exceed 57% with a corresponding minimum dry matter of 43%. All conditions were met in our experimental cheese. With no differences in compositional characteristics or in drying, aging, holding conditions, or initial inoculation level, the assumption is made that the only variable between cheeses is milk type. Variance between cheeses within vat cannot be controlled for and may contribute to any error observed as whole individual cheeses were sampled for bacteriological analysis. According to the FDA, Center for Food Safety and Applied Nutrition (CFSAN) Food Compliance Program for Domestic and Imported Cheese and Cheese Products (44), samples from both domestic and imported cheese samples must come from intact units from the same lot. In this study individual cheeses from the same lot were tested at each sampling point as opposed taking multiple samples from the same cheese over time to overcome heterogeneous pathogen distribution in cheese in addition to the fact that the fluid and soft texture of soft ripened cheeses make them difficult to cut and rewrap without altering the product.

During the initial 3-4 weeks of aging, populations of *L. monocytogenes* were below our detection limit. Some cells were likely transferred to the aging racks during the initial stages of ripening, as viable cells were detected through environmental sampling of the aging racks (data not shown). We were unable to determine, quantitatively, the extent of this transfer. The inability to enumerate was also observed by Ryser and Marth (37) when pasteurized milk Camembert-type cheese was surface inoculated. One strain failed to grow altogether but was detected through cold enrichment. However, in the aforementioned study *L. monocytogenes* was applied atop the *Penicillium camemberti* lawn on the surface of the cheese, an important factor

considering the organism likely had to penetrate the rind to enter the cheese before growth could occur. Decreases in populations on or near the surface of Camembert-type cheese during the initial stages of ripening have also been reported (1, 26). When examining the survival of *L. monocytogenes* in Camembert-type cheese at different pH values, Ryser and Marth (37) found populations decreased at pH 4.6 and failed to grow at pH 5.5. Cheese pH at dehooping (4.8-4.9) increases following the assimilation of lactate by *K. lactis* and *G. candidum*, decreasing the rind acidity, and through increased ammonia concentration within the cheese. Surface pH increases more rapidly through the proteolytic and peptidasic activity of ripening fungi, notably *P. camemberti* (23). In our study, growth initiated at pH values between 5.16 and 5.91, and once detectable, *L. monocytogenes* population growth paralleled the gradual increase in pH, in accordance with other studies (31, 33, 37). The delayed onset of growth is likely due to microbial injury incurred from the presence of salt, and the low pH (14) environment of the fresh cheese surface. When *L. monocytogenes* (strains Scott A, OH, and CA) was inoculated in cheese milk used for Camembert-type cheese manufacture, counts in wedge and interior samples decreased 10-100 fold during the first 17 days of ripening, an observation attributed to the low pH (<5.5) and storage temperature (15-16°C) (37). In a study by Gay and others (14), *L. monocytogenes* Scott A populations declined and were unable to grow in Richard's broth adjusted to pH 4.8 used to simulate Camembert cheese at the beginning of ripening. Increased lag times as a result of microbial injury have been previously reported for *L. monocytogenes* (3, 7, 18, 46). It has also been shown that the duration of the lag phase of *L. monocytogenes* under stresses such as salt (35) or conditions simulating soft cheese ripening (14) is also affected by inoculum size. Here

we observed that in some replicates at the low inoculation level, cells remained undetectable through day 70. It is possible that under very unfavorable growth conditions cells will die during the extended lag phase (32). Given a very small inoculum size, there is the possibility that due to heterogeneity in cellular stress responses within a population, all the cells in the inoculum could die and no growth would occur (32). It is also possible that a few cells could survive and initiate growth, as was observed in other replicates within the same inoculum set. It is likely that the presence of these null values and the variability in the duration of lag periods affected the growth potential between cheeses thus contributing to the high level of variation in the log mean CFU/g counts reported. The role of inoculation levels on the growth/no growth barrier is evidenced when we eliminate cheeses negative for *L. monocytogenes* from analysis and only analyze the positive counts. With no growth data eliminated there is no significant effect of initial inoculation level on the rate of growth, suggesting that once population growth commences, growth rates will be similar regardless of initial inocula. Storage of these cheeses at 4°C could also have restricted growth (1, 39). In a study by Little and Knochel (25), only the psychrotrophic pathogen *Yersinia enterocolitica* was able to grow when introduced as a post processing contaminant on stabilized Brie, whereas populations of both *Salmonella* and *Bacillus cereus* declined at 4°C and 8°C. Due to the difficulty and variability in the detection of *L. monocytogenes* from soft, mold ripened cheeses in general (34, 40), as well as at very low inoculation levels (20) especially when made from raw milk (34), samples testing negative by direct plating did not undergo standard enrichment procedures.

After 60 days of ripening, maximum population levels differed between the two initial inoculation levels employed. Ryser and Marth (37) observed 2-3 log cycles of growth in three strains of *L. monocytogenes* surface inoculated onto 10 day old Camembert-type cheese at 6°C. Maximum observed population levels of approximately 3-5 log CFU/g were similar to the 4.55 and 5.29 observed herein (high level) despite differences in initial inoculation level between studies. These contamination levels are also consistent with *L. monocytogenes* levels found in contaminated soft surface mold ripened cheeses at retail (17) and *Listeria innocua* levels under similar conditions of inoculation and ripening (39). Back and others (1) suggested an approximate 2 log increase in *L. monocytogenes* on the surface of laboratory manufactured Camembert-type cheese after 40 days at 3 and 6°C, although their results were not reported. Comparisons to data reported by Genigeorgis and others (15) are also difficult due to differences in methodology, storage conditions, and the fact that the authors reported results as log CFU/sample. Based on our results, the use of raw milk does not seem to provide enhanced protection, compared to pasteurized milk, when *L. monocytogenes* is introduced as a post processing contaminant in bloomy rind cheese. Similarly previous investigators have reported that the growth of *L. monocytogenes* during the manufacture and ripening of Camembert is unaffected by the use of a nisin producing starter or raw milk when compared to the use of pasteurized milk (33).

Based on our data and results previously reported, soft surface mold ripened cheeses support the growth of *L. monocytogenes* introduced post-processing when contaminated at levels ≥ 0.2 CFU/g, independent of the milk type used for manufacture. The use of pasteurized milk in the manufacture of soft surface mold ripened cheese does

not provide protection from *L. monocytogenes* introduced as post process contaminants. Despite low pH, lower moisture, and presence of salt, *L. monocytogenes* introduced as post processing contaminants were able to survive and grow on soft ripened cheeses held for ≥ 60 days. Moreover, the 60 day aging rule encourages extended holding of these cheeses which could inadvertently contribute to risk through the proliferation of psychrotrophic pathogens during refrigerated holding. The safety of cheeses within this category must be achieved through control strategies other than a 60 day holding period and revision of current federal regulations are warranted.

Under U.S. Department of Agriculture (USDA), Food Safety Inspection System (FSIS), manufacturers of ready to eat (RTE) products at high risk of *L. monocytogenes* contamination operate under mandatory Hazard Analysis Critical Control Point (HACCP) systems where products are considered adulterated not only if they contain *L. monocytogenes*, but if they have been in direct contact with a surface which is contaminated with *L. monocytogenes* as well. Producers must address control of *L. monocytogenes* in their HACCP plan, Sanitation Standard Operating Procedures (SSOP) or other prerequisite programs whose effectiveness is verified through frequent environmental and end product testing (41). Similar systems may be effective in preventing the contamination of high risk cheeses as well.

Canada is currently seeking to develop a policy to replace the 60 day aging requirement for soft and semi-soft cheeses following outbreaks of listeriosis in Canada in 2002 linked to cheeses made from pasteurized or heat-treated milk. Although not in line with domestic policy, the Canada Food Inspection Agency (CFIA) has allowed the importation of certified raw milk soft and semi-soft cheese from France. The

accompanying certificate verifies that the cheese was manufactured in compliance with French national standards and verifies that the products meet the microbiological criteria of both Canada and France.

It is clear based on epidemiologic evidence and the results of scientific investigation that neither pasteurization nor 60 days of aging are sufficient enough to ensure the safety of soft surface mold ripened cheese. It is expected that cheese safety can be better assured through a combination of stringent raw milk production and microbiological quality, improved process controls, the use of performance criteria, as well as aggressive environmental monitoring and finished product testing, than through current requirements.

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Figure Legends

Figure 1. Change in mean surface pH (\pm SD) of raw (\blacklozenge), and pasteurized (\blacksquare) milk cheese over time at the low (A) and high (B) inoculation level.

Figure 2. Change in mean interior pH (\pm SD) of raw (\blacklozenge), and pasteurized (\blacksquare) milk cheese over time at the low (A) and high (B) inoculation level.

Figure 3. Change in log mean counts (\pm SEM) of *L. monocytogenes* at the low (A) and high (B) inoculation level on the surface of raw (\blacklozenge), and pasteurized (\blacksquare) milk cheese during aging and holding.

Figure 4. Change in log mean counts (\pm SEM) of *L. monocytogenes* at the low (A) and high (B) inoculation level on the surface of raw (\blacklozenge), and pasteurized (\blacksquare) milk cheese during aging and holding using only non-zero positive data.

Tables

Table_ 1: Moisture non-fat substance (MNFS), salt in moisture phase (SMP), fat in dry matter (FDM), and protein values for raw (R) and pasteurized (P) soft surface mold ripened cheese.

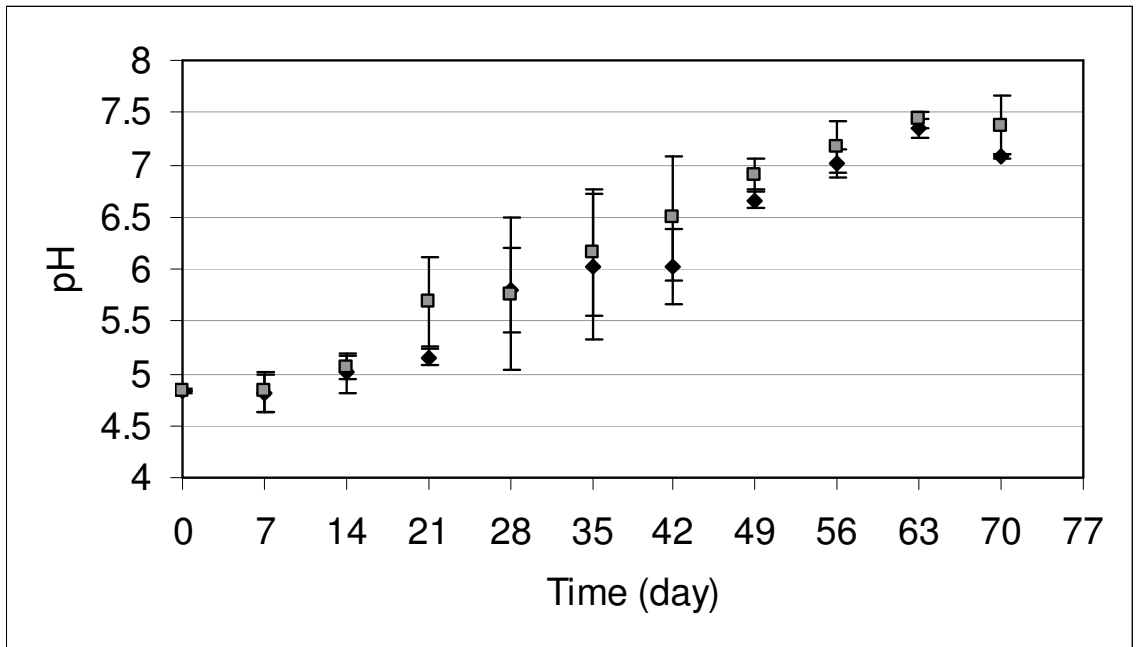
Trial	MNFS (%)	SMP (%)	FDM (%)	Protein (%)
1R	64.81	3.34	52	ND
1P	65.70	3.90	50	ND
2R	67.13	3.3	51	17.37
2P	69.65	3.10	51	17.81
3R	63.74	3.85	54	20.74
3P	66.15	3.53	53	20.39

ND: Not determined

Table_ 2: Number of *L. monocytogenes* positive samples by sampling day.

	Sampling Day									
	7	14	21	28	35	42	49	56	63	70
Raw High	0/3	0/3	0/3	1/3	1/3	2/3	3/3	3/3	3/3	3/3
Past. High	0/3	0/3	0/3	1/3	1/3	2/3	2/3	2/3	3/3	3/3
Raw Low	0/3	0/3	1/3	1/3	1/3	1/3	1/3	2/3	1/3	2/3
Past. Low	0/3	0/3	0/3	1/3	1/3	3/3	3/3	1/3	1/3	3/3

A.



B.

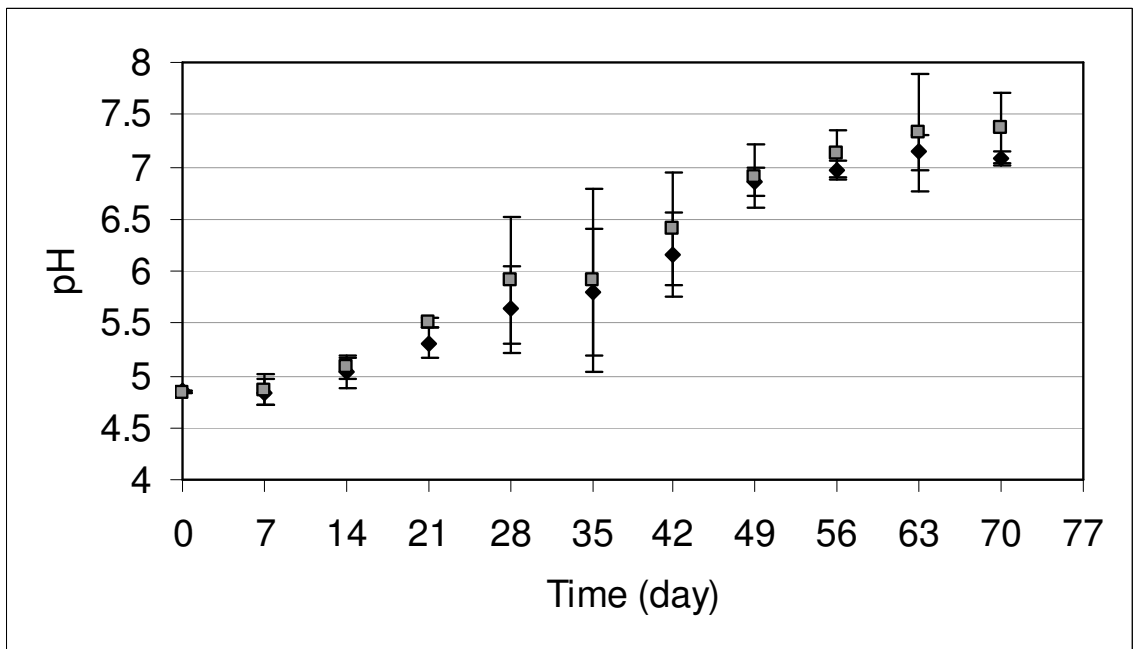
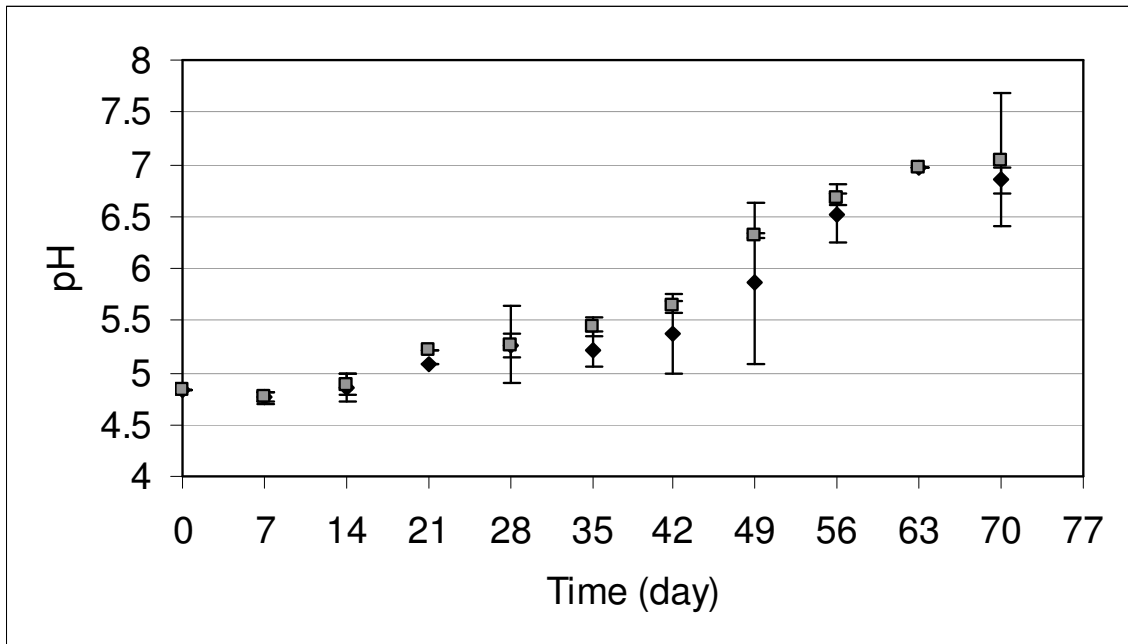


Figure. 1: Change in mean surface pH (+/- SD) of raw (♦), and pasteurized (■) milk cheese over time at the low (A) and high (B) inoculation level.

A.



B.

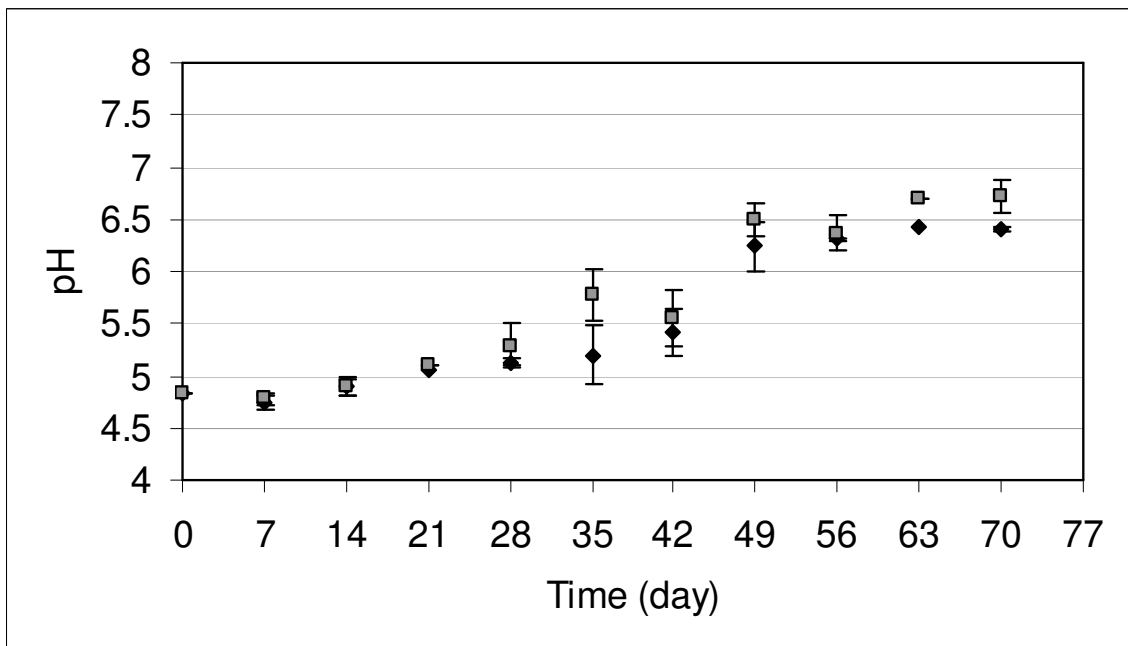
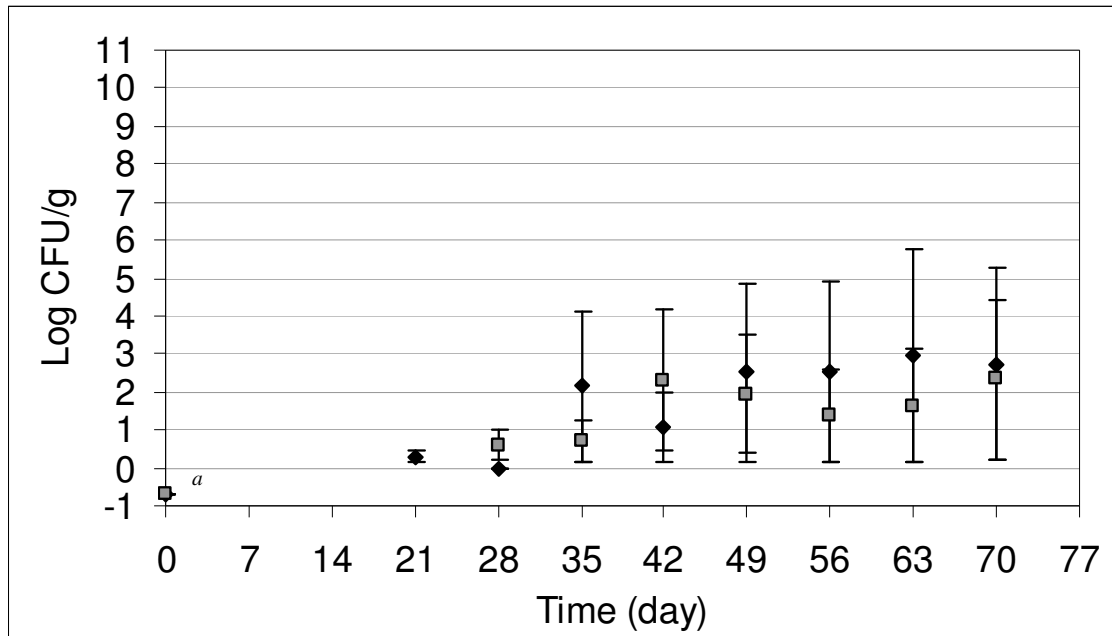


Figure. 2: Change in mean interior pH (+/- SD) of raw (♦), and pasteurized (■) milk cheese over time at the low (A) and high (B) inoculation level.

A.



B.

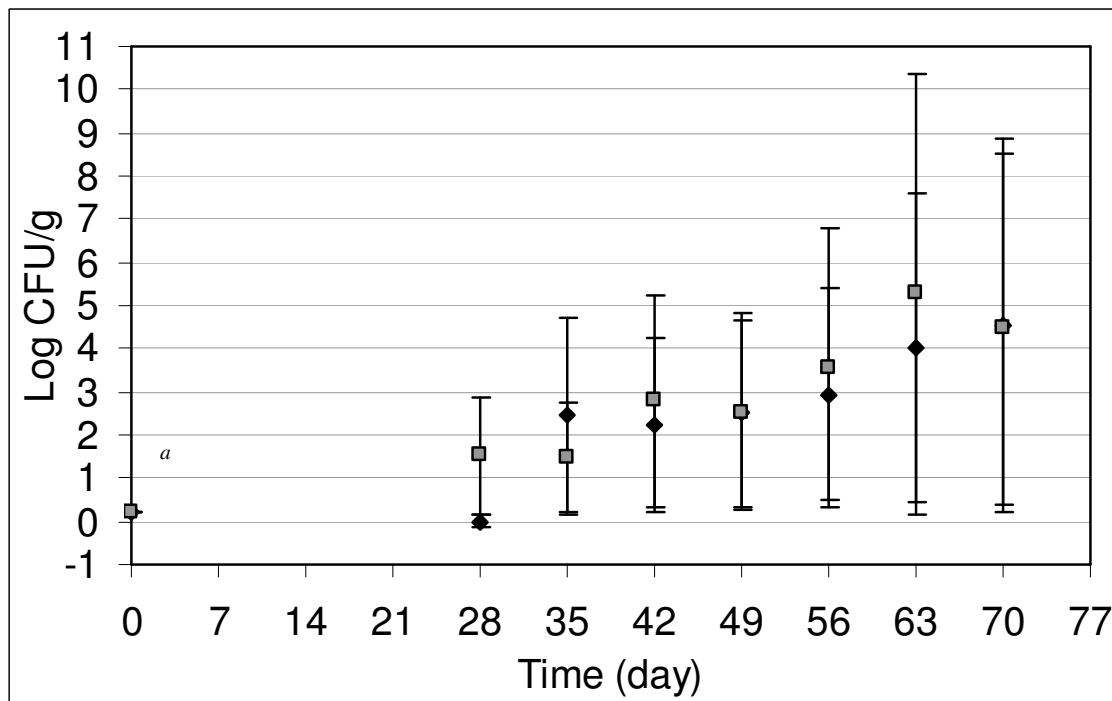
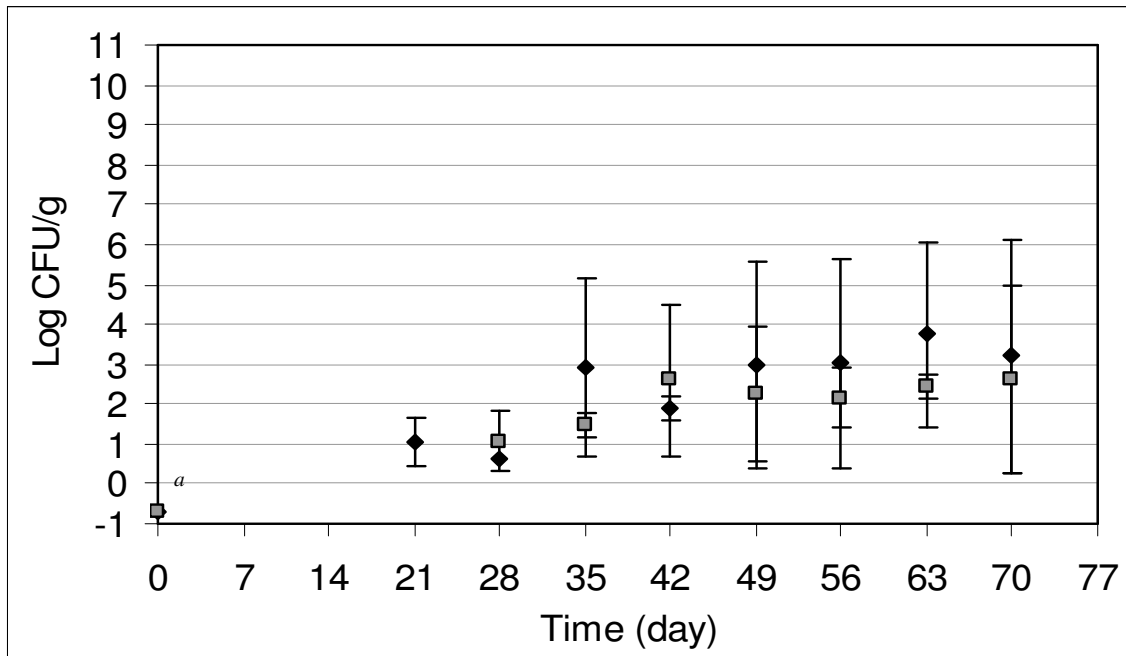


Figure. 3: Change in log mean counts (+/- SEM) of *L. monocytogenes* at the low (A) and high (B) inoculation level on the surface of raw (♦), and pasteurized (■) milk cheese during aging and holding. ^a: Data represents estimated inoculation levels (not actual counts).

A.



B.

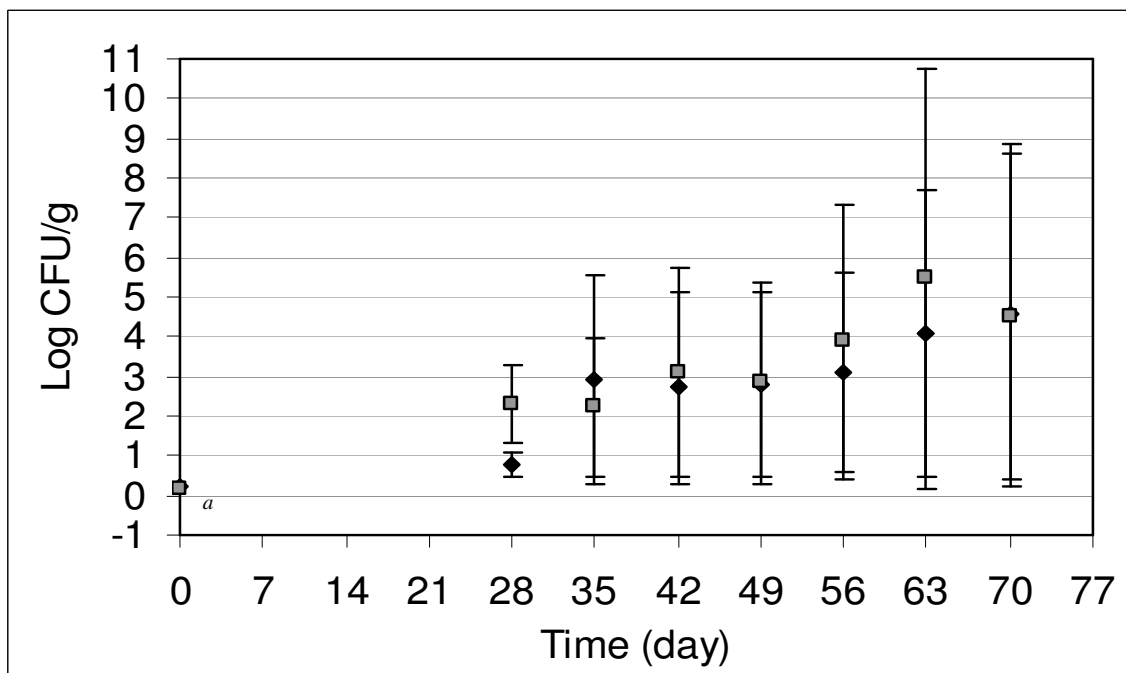


Figure. 4: Change in log mean counts (+/- SEM) of *L. monocytogenes* at the low (A) and high (B) inoculation level on the surface of raw (♦), and pasteurized (■) milk cheese during aging and holding using only non-zero positive data.

^a: Data represents estimated inoculation levels (not actual counts).

Chapter 3: Enhanced detection of *Listeria* spp. in farmstead cheese processing environments through dual primary enrichment, PCR and molecular subtyping.

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Listeria spp. in farmstead cheese processing environments.

Keywords: cheese, environmental sampling, *Listeria*, detection, PCR, ribotype.

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Abstract

The incidence and ecology of *Listeria* spp. in farmstead cheese processing environments was assessed through environmental sampling conducted in 9 different plants over a 10-week period. Environmental samples (n = 705) were examined for the presence of *Listeria* spp. using three detection/isolation protocols. The use of dual enrichment methods, which allowed for the recovery of injured *Listeria* spp. (mUSDA), identified more *Listeria* spp. positive samples with higher sensitivity than the standard USDA method. The addition of PCR to the mUSDA method identified the most *Listeria monocytogenes* positive samples, achieving greater sensitivity of detection while substantially reducing time. Overall, 7.5% of samples were positive for *Listeria* spp. yielding 710 isolates; 253 of which were subtyped by automated ribotyping to examine strain diversity within and between plants over time. The isolation of specific ribotypes did not appear to be affected by the enrichment protocol employed. Fifteen (2.1%) samples yielded *L. monocytogenes* isolates differentiated almost equally into ribotypes of lineages I and II. Of most concern was the persistent and widespread contamination of a plant with *L. monocytogenes* DUP-1042B, a ribotype previously associated with multiple outbreaks of listeriosis. Our results suggest that the extent of *Listeria* spp. contamination, notably *L. monocytogenes*, in farmstead cheese plants is comparatively low, especially for those with on-site farms. The results of this study also identified points of control for use in designing more effective *Listeria* spp. control and monitoring programs with a focus on ribotypes of epidemiological significance.

Consumer interest in artisan and farmstead cheeses is driving explosive growth of farmstead cheese operations throughout the United States. As many of these enterprises are small to very small operations, there is a need for focus on assuring the microbiological safety of cheeses produced on the farm. *Listeria monocytogenes* is an invasive intracellular bacterial pathogen capable of causing serious disease in both humans and animal species (11). Although readily inactivated by pasteurization, pasteurized milk and milk products have been implicated in outbreaks of listeriosis as the result of post-processing contamination (38). Effective environmental monitoring and control of *Listeria* spp. within processing plants, including farmstead cheese operations, is paramount in reducing cross contamination of RTE foods as *Listeria* spp. are generally regarded as indicator organisms for the presence of *L. monocytogenes*.

Listeria spp., including *L. monocytogenes*, occur widely in dairy farm environments (29) and are regularly isolated from dairy processing and cheesemaking environments (10, 20, 22, 23, 33, 34, 46, 48) where some strains of *L. monocytogenes* have been shown to persist, serving as sources of food product contamination (3, 18, 19, 20, 24). Dairy processing plants with an on-site dairy farm may have a higher risk of contamination than those plants without an adjacent farm where sources of *Listeria* spp. such as dairy cattle, raw milk and silage can subsequently contaminate processing environments (1, 33).

The U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) protocol for the isolation and identification of *L. monocytogenes* from environmental samples employs University of Vermont modified *Listeria* enrichment broth (UVM) for primary enrichment. The selectivity of UVM suppresses background

flora, allowing for the discrimination of low levels of *Listeria* spp. in highly contaminated samples. However, the selective agents in UVM may reduce the extent of recovery and subsequent detection of injured cells (2, 5, 37). The USDA-FSIS method (47) permits the additional use of *Listeria* Repair Broth (LRB) for duplicate samples to enhance detection by allowing for repair and recovery of cells injured by exposure to sanitizers (39). While neither enrichment broth is significantly better than the other singly (37), improved recovery of *Listeria* spp. from environmental samples through dual use of both LRB and UVM has been shown, providing the sensitivity of carrying out both procedures individually without increasing the workload (35).

The efficacy of *Listeria* spp. isolation from food and environmental samples (12, 16, 35, 50), including the isolation of specific subtypes (37) and genetic lineages (4), may also be affected by the enrichment procedures employed. The use of PCR has the potential to reduce pathogen detection time without sacrificing, and possibly enhancing, sensitivity and specificity (14). The BAX® PCR assay (Dupont™ Qualicon BAX® Q7) for screening *L. monocytogenes*, which generally performs as well or better than standard cultural methods (42), has been adopted by the USDA-FSIS and Health Canada (51) for the screening of *Listeria* spp. in environmental samples. In 2008, the USDA expanded their procedure to include BAX® PCR for the screening of *L. monocytogenes* (47).

As an adjunct to environmental sampling, subtyping of isolates can be an important tool in tracing contamination patterns within food processing plants thus aiding in the development of more effective intervention strategies (10, 44). While many subtyping methods exist, automated ribotyping is a sensitive and reproducible subtyping method commonly used for the differentiation of large sets of *L. monocytogenes* strains.

The standardization of the system makes it possible to combine and compare results from different instruments and between laboratories (44). Large data sets are also available for comparison in numerous publications and through the public database PathogenTracker 2.0 (available at: <http://www.pathogentracker.net>). Ribotypes have also been used to differentiate clones of *L. monocytogenes* as well as to predict lineage classifications (27) which vary in virulence within and between host species (21). As the predominant strains isolated from human listeriosis cases and outbreaks worldwide, lineage I strains are thought to have a higher likelihood of causing disease than those in lineage II, which are typically associated with food and food processing environmental isolates (15, 30).

The objective of this study was to fill data gaps in the literature regarding the prevalence of *Listeria* spp. in farmstead cheese processing environments. Specifically we sought to examine the incidence and distribution of *Listeria* spp., including subtypes, within and between small farmstead cheese processing environments over repeated sampling events. Additionally, we compared the efficacy of three protocols for the detection and isolation of *Listeria* spp., including specific subtypes, from environmental samples.

Materials and Methods

Environmental sample collection. Nine farmstead cheese plants were evaluated for the presence of *Listeria* spp. through environmental sampling conducted once a month over a three month period (September-November 2006) with the exception of Plant B which was only sampled on one occasion. One plant (plant F) was also revisited approximately one year later to better understand the duration of persistence within

contaminated plants. Environmental samples obtained at this follow up visit were analyzed using a single method (mUSDA/PCR; as described below). Data from this visit were not used in any analyses and resulting isolates were solely used for ribotype comparison. Processing plants manufacturing cheese from raw milk obtained from the farmer's own animals, on the farm where they were raised, were chosen based on geographic location, willingness to participate, and type of milk used for cheese manufacture (raw or pasteurized). All processors surveyed produced raw milk cheese and in some cases additional pasteurized milk products. Three technicians were each assigned three plants to sample throughout the duration of the study in an effort to control reproducibility and repeatability over time. Inherent variability due to differences in swabbing patterns, as well as angle and degree of pressure applied between technicians could not be controlled for (26).

Thirty environmental sites per plant, sampled at each of the three visits, were chosen for sampling consisting of approximately 15 food contact surfaces (FCSs) and 15 non-food contact surfaces (NFCSSs). Sites were selected based on recommendations from published literature (45) and plant personnel in order to include areas of particular concern. Sites were also determined based on conditions in the plant on the day of sampling, thus some sites were not available for resampling upon subsequent visits. Sites positive for *Listeria* spp. in previous sampling events were, however, resampled on each subsequent visit to determine frequency of contamination and persistence of strains over time.

Prior to pre-production sanitation, sterile environmental sampling sponges (EnviroSponge™, Biotrace International, Bridgend, United Kingdom) were hydrated

with 10 ml Dey-Engley (D/E) broth (RediTube™, Biotrace) to neutralize disinfectants. Excess buffer was removed from the hydrated sponge prior to surface sampling. Using sterile gloves, large flat surfaces were sampled by wiping a 2 x 3ft area in a horizontal direction. The sponge was then turned over and the opposite side and end was used to sample the same area in a vertical direction. For drain samples, all accessible surfaces were wiped including sidewalls and grates. Pooled water samples were obtained by wiping the surrounding edge of the pool while allowing the sponge to absorb a portion of the liquid. In all cases, following sampling, sponges were returned to the sterile bag, hand mixed for 1min and placed on ice for transport to the laboratory. Data loggers (SP125, Dickson, Addison, Ill.) recorded time and temperature during transport to assure maintenance of correct temps (<4°C) prior to arrival at the laboratory. Samples were processed within 4h of collection to preserve microbial viability and prevent multiplication (26). Upon arrival in the laboratory 10ml of Buffered Peptone Water (BPW) was added to each bag and mixed by hand for 1min. Ten ml of liquid sample eluted from the sponge was split between 2 separate enrichment broths (UVM and LRB) at 1:10 dilutions for the three different detection/isolation protocols described below, and the sponge was discarded.

Protocols for the detection and isolation of *Listeria* spp. Protocols used in this study included: USDA-FSIS (USDA; 47), modified USDA-FSIS (mUSDA) as previously described (35) with minor alterations, and mUSDA with the addition of PCR (mUSDA-PCR).

For the USDA method, samples underwent single primary enrichment in UVM broth (Becton Dickinson. Franklin Lakes, N.J.) incubated at $30 \pm 2^{\circ}\text{C}$ for 22 ± 2 h.

Following primary enrichment, 0.1 ± 0.02 ml was added to 10 ± 0.5 ml of Fraser broth (FB) with ferric ammonium citrate added at 0.5g/liter (Becton Dickinson) and incubated at $35 \pm 2^\circ\text{C}$ for 26 ± 2 h. After 26 ± 2 h, tubes were examined for darkening resulting from esculin hydrolysis. If FB darkening was evident, 0.1ml of FB was spread onto Modified Oxford selective *Listeria* agar (MOX; Becton Dickinson) using a sterile loop and incubated at $35 \pm 2^\circ\text{C}$ for 26 ± 2 h. If no FB darkening was evident, tubes were re-incubated at $35 \pm 2^\circ\text{C}$ until a total incubation time of 48 ± 2 h was reached. After 48h, FB tubes were reexamined for darkening, and positive samples were streaked onto MOX and incubated for 26 ± 2 h at $35 \pm 2^\circ\text{C}$. Following incubation, MOX plates were examined for suspect *Listeria* growth (small colonies, approximately 1 mm, surrounded by a zone of darkening). A swipec of suspect growth representing at least 20 colonies was then streaked to another MOX plate for isolation and incubated at $35 \pm 2^\circ\text{C}$ for 26 ± 2 h. If no suspect colonies were evident, MOX plates were re-incubated until a total incubation time of 48 ± 2 h was achieved. Five typical colonies from each plate were individually purified on tryptic soy agar with 0.6% yeast extract (TSAYE; Becton Dickinson) and incubated for 24h at $35 \pm 2^\circ\text{C}$ for further biochemical identification.

Dual primary enrichment for the mUSDA method was carried out separately in both UVM and LRB (5) incubated for 24h at 30°C . Filter sterilized solutions (0.45 μm nitrocellulose filter; General Electric Water and Process Technologies) resulting in 40mg/liter of nalidixic acid-sodium salt (Sigma-Aldrich. St. Louis, Mo.), 50mg/liter of cyclohexamide (Sigma-Aldrich) and 15 mg/liter of acriflavine-HCL (Sigma-Aldrich) were added to LRB following 4-5h of incubation at 30°C . For secondary enrichment, 0.1ml each of UVM and LRB were inoculated into 10 ± 0.5 ml FB and incubated at $35 \pm$

2°C for 26 ± 2h. Following inoculation of FB, the mUSDA procedure followed that of the USDA as previously described.

For mUSDA-PCR, samples underwent dual primary enrichment in both LRB and UVM as described for the mUSDA with the following modification. For secondary enrichment, 0.1ml each of UVM and LRB were inoculated into 9.9ml MOPS Buffered *Listeria* Enrichment Broth (MOPS-BLEB) and incubated for 18-24h at 35 ± 2°C. Following secondary enrichment, all MOPS-BLEB samples were screened for *Listeria* spp. using the Genus *Listeria* PCR assay for the Dupont Qualicon BAX® Q7 (BAX® PCR; DuPont Qualicon, Wilmington, Del.) according to the manufacturer's instructions. Although a positive control is built into the BAX® PCR tablets, both positive and negative MOPS-BLEB controls were run with each batch. Lysates deemed positive for *Listeria* spp. by PCR were additionally analyzed with the *L. monocytogenes* PCR assay for BAX®. One pure isolate from each culture-positive, PCR-negative sample was tested using the BAX® system to verify false negative results were not due to failure of the BAX® system to recognize all *Listeria* spp. isolates (17). Isolation and detection of suspect colonies from MOPS-BLEB enrichments (0.1ml) on MOX and TSAYE followed the USDA and mUSDA methods.

Purified isolates from TSAYE underwent further biochemical identification based on Gram reaction, umbrella motility, catalase activity and acid production in carbohydrate fermentation medium containing either 0.5% xylose, mannitol or rhamnose with bromocresol purple indicator. Characteristic colonies were also streaked onto CHROMagar™ *Listeria* (CHROMagar Paris, France) to differentiate *L. monocytogenes* from *L. innocua*. All isolates were maintained on TSAYE slants stored at 4°C following

incubation at 35°C for 24h. Cultures from TSAYE slants were streaked to Brain Heart Infusion Agar (BHIA) plates and incubated for 24h at 35°C prior to ribotyping.

Ribotyping: In order to examine subtype diversity between plants, as well as within a given sample, *Listeria* spp. isolates were subtyped using the automated Riboprinter® Microbial Characterization System (Qualicon Inc.) according to the manufacturer's instructions using the restriction endonuclease *EcoRI* (Qualicon Inc.). Proprietary RiboExplorer® software (V.2.0.3121.0) normalized resulting fragment pattern data for band intensity and relative position and automatically assigned DuPont identifications (DUP-ID; e.g., DUP-19171). All patterns and DUP-ID assignments were confirmed by visual inspection as previously described (15). If an assigned DUP-ID included more than one distinct ribotype pattern then each pattern was designated with an additional letter (e.g., DUP-1062B and DUP-1062C); these IDs were assigned to be consistent with the nomenclature used by the Food Safety Laboratory at Cornell University (15, 18, 21, 22, 29, 30, 36, 40, 41). Resulting DUP-IDs from *L. monocytogenes* isolates were also used to assign isolates to one of three previously described lineages (30, 40, 41, 44, 52). Ribotypes were considered persistent when isolates of the same DUP-ID were recovered from the same environmental site in sequential samplings. Similarly, DUP-IDs recovered from the same plant in more than one sampling event, regardless of specific site, were considered resident subtypes.

Data analysis. Mean frequencies of *Listeria* spp. and *L. monocytogenes* contamination between FCSs and NFCs were compared via a two tailed Student's t-test. Results with P values <0.05 were considered significant. Comparison of isolation and detection methods was based on the parameters as described by Flanders and colleagues

(12): true positive (TP), positive test with confirmed *Listeria*; false positive (FP), positive test without confirmed *Listeria*; true negative (TN), negative test without confirmed *Listeria*; false negative (FN), negative test with confirmed *Listeria*. Calculated values were used to evaluate and compare methods using the following equations:

$$\text{Sensitivity (\%)} = 100 * TP / (TP + FN)$$

$$\text{Specificity (\%)} = 100 * TN / (TN + FP)$$

$$\text{Predictive value of a positive test (\%)} = 100 * TP / (TP + FP)$$

$$\text{Predictive value of a negative test (\%)} = 100 * TN / (TN + FN)$$

$$\text{Accuracy (\%)} = 100 * TP + TN / (TP + TN + FP + FN)$$

Results

Incidence of *Listeria* spp. in farmstead cheese processing environments. A total of 705 environmental samples were collected from farmstead cheese processing plants. One or more species of *Listeria* was identified in 53 of the 705 (7.5%) samples. As shown in Table 1, the mUSDA method identified 51 of 53 positive samples, with only two false negatives. The use of PCR, as opposed to standard cultural identification, produced an additional false negative identifying 50 of the 53 positive samples. The USDA method, which only identified 41 *Listeria* spp. positive samples, produced 12 false negative results, thus failing to identify 22.6% of positive samples. No false positive (FP) results were obtained from any of these methods. In all nine plants, one or more samples collected during one or more visits were positive for *Listeria* spp. for an overall plant contamination rate of 100%. Frequency of isolation from environmental

sites within individual plants, as shown in Table 3, ranged from 1.2% to 19.8% with a mean frequency of 7.3%.

L. monocytogenes was isolated from 15 of the 705 environmental samples for an overall prevalence of 2.1%. The mUSDA/PCR method displayed the highest sensitivity identifying 14 of 15 *L. monocytogenes* positive samples, whereas the standard cultural version of the mUSDA method detected only 11 (Table 1). The mUSDA method produced four false negatives while the mUSDA/PCR method had only one. The USDA method again had the highest number of false negative results identifying only 9 of the 15 *L. monocytogenes* positive samples with the lowest sensitivity of 60%. Overall, methods employing dual enrichment procedures (mUSDA with and without PCR) identified four unique *L. monocytogenes* positive samples missed by the USDA method, which in turn identified one unique sample. Cultural procedures failed to recover *L. monocytogenes* from the MOPS-BLEB enrichments of four PCR positive samples. As a result, the mUSDA/PCR method for *L. monocytogenes* yielded four false negative results with 89.5% specificity, compared to the standard cultural methods at 100%. Of the nine plants sampled, *L. monocytogenes* was recovered from four, with frequencies of isolation within positive plants ranging from 1.1% to 10.3% (Table 2) with a mean frequency of 4.4%, and an overall mean frequency of 1.9% for all plants.

Distribution and diversity of *Listeria* spp. isolates. From 53 positive samples, confirmation of 5 isolates per positive method yielded 710 isolates of *Listeria* spp. *L. innocua* was the most frequently isolated species accounting for 70.8% (503/710) of isolates recovered from 73.6% (39/53) of positive samples. Subtyping by automated ribotyping differentiated 96 *L. innocua* isolates into seven distinct ribotypes (DUP-1006,

DUP-1010, DUP-1019, DUP-1005, DUP-1007, DUP-1009 and DUP-1020). *L. innocua* DUP-1005 (32.3% of isolates) and DUP-1006 (37.5% of isolates), isolated from five (55.6%) and three (33.3%) plants, respectively, were the most common subtypes. Although only 12 of the 96 isolates (13%) were identified as DUP-1009, this ribotype was found in three separate plants. *L. innocua* ribotypes DUP-1007, DUP-1010, DUP-1019 and DUP-1020 were both rare and unique to individual plants.

L. monocytogenes accounted for 20% (142/710) of the isolates from 15 of the 53 (28.3%) sites positive for *Listeria* spp. Based upon visual examination of fragment banding patterns, isolates of *L. monocytogenes* were differentiated into five separate ribotypes including one of lineage I (DUP-1042B), three of lineage II (DUP-1039C, DUP-1030B, DUP-1045A) and one of lineage III (DUP-10144). Of the five ribotypes, only DUP-1030B and DUP-1039C were isolated from multiple plants. Although DUP-1039C only represented 4% of the total *L. monocytogenes* isolates, this ribotype was found in three of the four *L. monocytogenes* positive processing plants while DUP-1030B, a more common subtype (37.3% of isolates), was recovered from two. Ribotype DUP-1042B, the most common *L. monocytogenes* subtype isolated (55.6% of isolates), as well as DUP-10144, were unique to a single processing plant (plant F). All isolates of DUP-1045A, recovered solely from plant G, were catalase negative.

L. innocua and *L. monocytogenes*, the only species isolated together, were isolated alone in 29 (54.7%) and 9 (17%) of 53 positive sites, respectively, and together in six (11.3%). PCR positive, non-culture confirmed *L. monocytogenes* were detected with *L. innocua* in an additional four samples (7.5%). Of the six culture confirmed samples, four, obtained from the same plant, consisted of the same ribotype combination

(*L. monocytogenes* DUP-1042B and *L. innocua* DUP-1006; Table 3). *L. monocytogenes* DUP-1039C was isolated with either *L. innocua* DUP-1009 or DUP-1006 in the remaining two instances (Table 3). Additionally, seven samples, including two *L. monocytogenes* positive samples from plant F and five *L. innocua* positive samples from plant I, yielded more than one ribotype of the same species (Table 3). One such sample, a floor squeegee, yielded three different *L. innocua* ribotypes. Although uncommon, 35 of the 710 isolates (5%) recovered from three (33.3%) plants were identified as *L. seeligeri* differentiated into two ribotypes, DUP-1064 and DUP-1065. Thirty of the 710 isolates (4%) recovered from a single plant were confirmed as *L. welshimeri* of a single ribotype (DUP-1074). All *Listeria* spp. subtypes were consistently isolated by each of the three detection and isolation procedures with the following exceptions: *L. monocytogenes* DUP-10144 was solely isolated by the mUSDA and mUSDA-PCR methods, *L. seeligeri* DUP-1064 was isolated solely by the mUSDA and USDA methods and *L. innocua* DUP-1010 was only isolated by the USDA method.

Frequency and patterns of contamination by environmental sampling site.

Six of 351 (1.7%) FCS and 46 of 354 (13%) NFCS samples were positive for *Listeria* spp. while two FCS (0.6%) and 13 (3.7%) NFCS samples were positive for *L. monocytogenes* based on subsequent confirmation. Frequency of *Listeria* spp. contamination for FCS samples ranged from 0 to 7.1%, with a mean of 1.6%, and 2.5 to 31.8%, with a mean of 12.6% for NFCS samples. The mean frequency of *Listeria* spp. contamination of NFCS samples was significantly higher than that for FCSs ($p = 0.006$) as determined by two tailed Student's t-test. Frequency of *L. monocytogenes* contamination for FCS samples within individual plants ranged from 0 to 4.9%, with a

mean of 0.5% and 0 to 15.2% with a mean of 3.2% for NFCS samples. Differences in mean *L. monocytogenes* contamination frequencies between NFCS and FCS samples were not significant ($p = 0.08$). Contaminated drains were found in a majority (66.7%) of plants and constituted 45.3% and 46.6% of the *Listeria* spp. and *L. monocytogenes* positive samples, respectively. Other NFCS samples contaminated with *Listeria* spp. included floors (18.8%), squeegees (7.5%), pooled water (5.7%), water hose nozzles (5.7%), the wheels of carts (3.8%) and a wall adjacent to pooled water (1.8%). Contaminated FCS samples included cheese knives (3.8 %) cut and wrap tables (3.8 %), as well as ladles and buckets used to transfer curds to cheese hoops (3.8 %). The incidence of *L. monocytogenes* by sample site was similar to that of *Listeria* spp. with a few exceptions.

Subtype data, shown in Table 3, helps illustrate contamination patterns within individual plants. Four of the nine (44.4%) plants (D, F, H, and I) harbored persistent ribotypes sequentially isolated throughout the study. Persistence was generally observed in drains, where approximately half (46.2%) of drains positive in at least one sampling, yielded the same ribotype in subsequent samplings. *L. innocua* DUP-1019 and DUP-1005 were persistent drain contaminants in plants D and H, respectively. *L. innocua* DUP-1006, a persistent drain isolate in plant I, was consistently isolated from all three drains in plant F, one of which harbored *L. monocytogenes* DUP-1042B over subsequent samplings as well. DUP-1042B was also recovered from this same drain in a follow up sampling conducted one year later. In addition to drains, floor squeegees and hose nozzles also harbored persistent subtypes, albeit less commonly.

In addition to the presence of persistent strains isolated from specific sites, these plants appear to contain resident subtypes present in a wide range of sample sites. In some cases subtypes were only observed in a single sampling event. For example, *L. welshimeri* DUP-1074 isolated from both floor squeegee and aging room floor samples obtained in the first sampling of plant E was not detected in subsequent samplings (Table 3). In other instances, subtypes isolated from a specific site in one visit were isolated from nearby sites in subsequent visits. This can be seen in plant G where *L. monocytogenes* DUP-1030B was isolated from pooled water and drain samples from the initial visit and from a different, yet nearby drain from the next. In other more heavily contaminated plants, specific subtypes isolated from multiple samples taken throughout the plant better demonstrates possible contamination patterns. In plant F, *L. monocytogenes* DUP-1030B was isolated from a drain on visit one, the floor squeegee on visit two and a bucket used to fill hoops (FCS) on visit three. *L. monocytogenes* DUP-1042B was also isolated from a drain on visits one and two, as well as pooled water, the nozzle of a hose, and the bucket used to fill hoops (FCS) on visit two and finally the hallway floor and the cut and wrap table (FCS) on visit three. Although similar sites were again positive for *Listeria* spp. on this follow up visit, they yielded different subtypes (*L. innocua* DUP-1009 and DUP-1010) not identified previously. Interestingly, one year later *L. monocytogenes* DUP-1042B was isolated from plant footwear, a site not sampled in previous sampling events. In plant I, *L. innocua* DUP-1005 was isolated from the aging room drain, aging room floor and the floor squeegee on visit one, the cut and wrap table (FCS) on visit two, and the floor of the entrance on visit three. *L. innocua* DUP-1007 and DUP-1006 were also common to the drains (production room) and floor

squeegee on visit one and the cheese knife (FCS) and entrance floor on visit two (Table 3).

Discussion

The incidence and distribution of *Listeria* spp., including *L. monocytogenes* in farmstead cheese processing environments over a 10-week period using three different detection protocols was examined. Consistent with previous studies (12, 31, 35), methods with dual primary enrichment incorporating a repair step to recover injured *Listeria* (mUSDA with and without PCR), displayed the highest detection sensitivity for both *Listeria* spp. and *L. monocytogenes*. The combination of UVM and LRB in dual primary enrichment resulted in the identification of 12 additional *Listeria* spp. positive samples, including six containing *L. monocytogenes*, missed by single primary enrichment in UVM per the USDA method. False negatives observed in the USDA method are not likely the result of false negative FB (*Listeria* spp. present with no blackening; 12; 33) as samples in both the mUSDA and the USDA methods underwent secondary enrichment in FB. Since five of the six false negative results for *L. monocytogenes* attributed to the USDA method consisted of the lineage I ribotype DUP-1042B, it is possible that the selective growth advantage of *L. innocua* over lineage I strains when co-inoculated in UVM broth may have masked the presence of *L. monocytogenes* (4). Alternatively, the increased sensitivity of dual enrichment methods could have resulted from the repair and recovery of injured *Listeria* spp. in LRB, a process which would be inhibited by the selectivity of enrichment in UVM alone. It is this selectivity that likely allowed for the identification of the unique positive sample

detected by the USDA method missed by both dual primary enrichment based methods (37). It is unlikely that this specific isolate was specifically favored by enrichment in UVM broth as numerous isolates of the same ribotype (*L. innocua* DUP-1006) were obtained by each method. False negative results, independent of method, may have resulted from uneven distribution of *Listeria* cells to primary enrichment broths when the original sample was split, especially in samples with low levels of contamination.

The addition of PCR to the mUSDA method not only increased the sensitivity of *L. monocytogenes* detection but also shortened time to results. Although the mUSDA outperformed its PCR counterpart in the detection of *Listeria* spp., the difference consisted of only one positive sample. Two of the three false negatives observed in the mUSDA-PCR method for Genus *Listeria* were also negative through standard culture procedures (mUSDA) implicating enrichment as opposed to PCR as the root cause. The unique *L. monocytogenes* isolate (DUP-1039C) detected by the USDA method could have been missed by the dual enrichment procedures as a result of competitive overgrowth by *L. innocua* in LRB thus masking the detection of *L. monocytogenes* present at lower levels. This may be supported by the fact that *L. monocytogenes* constituted only one of the five isolates obtained from this sample via the USDA method.

Although the mUSDA/PCR method identified the most *L. monocytogenes* positive samples, it also displayed the lowest specificity for *L. monocytogenes* compared to the standard cultural methods as a result of four false positive results. Because of the dilution steps and the detection limits of the BAX® system, it is unlikely that false positive results are due to detection of non-culturable cells or DNA from dead cells (17). It is more likely that PCR has a higher sensitivity than the standard cultural methods used

in our study. Based on subsequent PCR detection of the three isolates missed by BAX® screening (*L. innocua* DUP-1006, *L. seeligeri* DUP-1064 and *L. monocytogenes* DUP-1039C), the false negative results from the mUSDA-PCR method were not due to the failure of the BAX system to recognize the specific isolate or ribotype.

Standard cultural procedures (USDA and mUSDA) produced the most false negative results for *L. monocytogenes*. One possible explanation is the competitive overgrowth of other *Listeria* spp. during selective enrichment where, when present together, *L. innocua* may outgrow *L. monocytogenes* in UVM and FB and on selective agar media (2, 9, 32, 37). The presence of high numbers (10^6 - 10^7 CFU/ml) of other *Listeria* spp., which may mask culture identification of *L. monocytogenes*, does not seem to influence PCR detection (28). In this study we purified five typical colonies per agar plate for biochemical analyses and species identification. It has been suggested that 10 colonies per agar plate be examined to ensure identification is based on equal representation of all species that may be present (37). The use of chromogenic agar as primary plating media may also aid in the identification of low levels of *L. monocytogenes* amidst higher proportions of other *Listeria* spp.

The combined results of all methods identified *Listeria* spp. in one or more environmental sites of each farmstead cheese plant participating in our study in at least one sampling event. Almost half (44.4%) of these plants also contained *L. monocytogenes*. The combined data from the repeated sampling of plants as opposed to a single sampling presumably increased the likelihood of a farm obtaining a *Listeria* spp. positive sample, including *L. monocytogenes*, thus resulting in a comparatively higher plant incidence rate. In a similar study, Pritchard and others (33) also recovered *Listeria*

spp. from 100% of dairy processing plants with a farm present compared to 76.2% of plants without an on-site farm. In a survey of California milk processing plants, 7.7% to 42.9% were positive for *Listeria* spp. depending on the type of plant examined, with cheese plants on the lower end of the range with 9.8% and 4.9% positive for *Listeria* spp. and *L. monocytogenes*, respectively (6). Although this suggests the incidence of *Listeria* spp. in the farmstead cheese processing plants is higher than that of other cheese plants, these data do not reflect the overall extent of contamination within plants or the variation between them.

Although listeriae were detected in each plant at least once in our study, the overall incidence of *L. monocytogenes* and *Listeria* spp. in environmental samples (n = 705) was only 2.1 and 6.7%, respectively. Similarly, the mean frequency of *L. monocytogenes* and *Listeria* spp. isolation was quite low at 1.9 and 7.3%, respectively. These contamination levels are lower than those reported in a survey of a farmstead dairy processing plant in New York state manufacturing cultured dairy products, including cheese, as well as those reported for Latin-style fresh cheese processing plants (18, 22). Even higher incidence rates have been reported for dairy processing plants with (34.2-57.9%) or without (38.5%) farms present where mean frequencies of isolation indicated that those with adjacent farms are at higher risk of contamination (12, 33). While the authors suggested that improved testing methodology contributed to the higher isolation rates when compared to previous surveys (33), we identified far fewer *Listeria* spp. positive samples using similar methodology. In comparison with the results of various surveys, our results seem to support the suggestion that processing plants manufacturing

cultured dairy products, including cheese, may be contaminated to a lesser extent than fluid milk processing plants and those manufacturing frozen milk products (6, 23, 33).

The isolation of *L. monocytogenes* from less than half of the plants positive for other species questions whether monitoring for *Listeria* spp. is a good predictor of *L. monocytogenes* contamination as suggested by Kabuki and colleagues (22). It is important to note, however, that all processing environments from which *L. monocytogenes* was isolated were also positive for other *Listeria* species. Consistent with previous studies (8, 20, 33, 34), *L. innocua*, followed by *L. monocytogenes* were the most prevalent species isolated. Overall site contamination rates for *L. innocua* (54.7%) and *L. monocytogenes* (17%) singly, or both species together (11.3%), is also similar to those previously reported (12), although the incidence of *L. innocua* is lower in the present study. Walker and others (49) reported that only a single *Listeria* species was recovered from 66% of frozen milk product plants surveyed compared to less than half (44.4%) of the farmstead cheese plants surveyed here, despite our assumption that repeated sampling of plants over time increases likelihood of a farm obtaining more than one *Listeria* spp. Single species appear to predominate in a given site as only a single *Listeria* spp. was isolated from 88.7% (47 of 53) of positive samples, analogous to the 94.6% reported by Walker and colleagues (49). Among individual samples yielding more than one species in our study, *L. monocytogenes* was solely isolated in conjunction with *L. innocua*.

Although the subtyping of *L. innocua* facilitated the tracing of contamination patterns in plants, ribotype information is rarely reported in the literature or included in public databases such as PathogenTracker 2.0 (available at:

<http://www.pathogentracker.net>). Similarly, reports regarding the isolation of *L. monocytogenes* DUP-10144, a lineage III strain, are scant with only one report found related to food (44). Lineage III strains, while capable of causing human disease, are typically associated with animal clinical cases (36). It has been suggested that the rarity of human listeriosis cases and outbreaks may be a function of limited human exposure resulting from poor survival in food and food processing environments, as opposed to reduced virulence (36). The limited isolation of lineage III strains observed here seems to support this given the proximity to animals and other possible farm sources of these strains. Aside from DUP-10144, the remaining *L. monocytogenes* ribotypes isolated in the present study have been previously associated with dairy processing plants, including those manufacturing cheese, and appear to be common to farm environments (29, 40, 41). In an analysis of the distribution of *L. monocytogenes* ribotypes among isolates from various sources, Sauders and colleagues (40) found that the prevalence of lineage II strains, DUP-1030B, DUP-1039C and DUP-1045A, was higher in farm isolates when compared to food and/or human clinical cases, which could result from strain adaptation to the ruminant host (DUP-1039C) and/or the farm environment (DUP-1045A and DUP-1039C; 29). This suggests that the contiguous farms associated with these plants may serve as a source of *Listeria* spp. contamination, although this is an area that requires further investigation. While DUP-1030B has been previously isolated from feces, water, and soil samples as well as sporadic human and bovine clinical cases (29, 40, 41), this ribotype does not appear to be commonly associated with food processing environments. An isolate of DUP-1030B was, however, isolated from a farmstead cheese and yoghurt processing plant (18). *L. monocytogenes* DUP-1039C and DUP-1045A have both been

isolated from cheese processing environments including a plant in Vermont (22) where the isolation of *L. monocytogenes* DUP-1045A from multiple environmental samples, as well as a sample of finished product highlights the role of the environment in the recontamination of finished products. DUP-1045A has also been isolated from unrelated cheeses including samples of fresh soft and internal mold ripened varieties (13). *L. monocytogenes* DUP-1039C, isolated from numerous human cases (41), appears to be overrepresented among farm and food isolates when compared to those from human clinical cases (15, 40).

L. monocytogenes DUP-1042B was not only the most commonly isolated ribotype in this study, but was also the only lineage I subtype identified. In general, lineage I strains are more commonly associated with human clinical isolates than those in lineage II whose strains are typically associated with isolates from food and food processing environments (15, 40, 41, 44). Furthermore, strains of lineage I tend to produce significantly larger plaques in cell culture assays than strains of other lineages (15, 52) suggesting that they have a higher likelihood of causing disease (15). The isolation of DUP-1042B is of utmost concern as strains of this subtype, including epidemic clone IV, are responsible for major outbreaks of human disease including those linked to the consumption of raw vegetables in Boston, pâté in the United Kingdom, ice cream in Philadelphia, and pasteurized milk in Massachusetts (7). Although *L. welshimeri* and *L. seeligeri* were also isolated in this study, their detection in dairy processing plants is rarely described in the literature. Based on the observance that *L. welshimeri* and *L. seeligeri*, when present, were only isolated from a single sampling event, it is possible

that the limited isolation of these species is a function of an inability to persist in food processing environments.

The range of sites from which *Listeria* spp. were isolated demonstrates the widespread contamination that can occur within processing plants. As previously noted by Pritchard and colleagues (34), our results indicate that NFCs such as drains and floors are more commonly contaminated with *Listeria* spp. than those in contact with food such as processing equipment. In agreement with previous surveys (8, 18, 22, 49), drains were the most frequently contaminated environmental site with 24 of 69 (34.8%) drain samples positive. This, and the subsequent contamination of nearby sites, supports the suggestion that drains may be useful in assessing the presence or absence of *Listeria* spp. in a general location of a plant (6). Other positive sites observed here, including pooled water and floors, are also not uncommon in dairy processing plants (8, 18, 22, 49). Recovery of *Listeria* spp. from NFCs in direct contact with water suggest that reducing the amount of pooled water will help in controlling pathogens in the dairy plant as previously suggested (34). Furthermore, *L. monocytogenes* can survive in aerosols created by the use of high pressure water hoses and brushes during cleaning, thus transferring cells from reservoirs such as drains and floors throughout the environment (43) including FCSs. Although wood is considered as a probable site harboring *Listeria* spp. (25), no *Listeria* spp. were isolated from aging shelves, hoops, or any other site made from or containing wood in this study. A large portion of isolates were, however, obtained from sites manufactured from rubber including hose nozzles and floor squeegee heads.

The recovery of multiple subtypes from floor squeegee samples suggests that cleaning tools may serve as harborage sites (3) and sources of dissemination, contaminating other sites such as floors, pooled water and drains. It is also possible that the squeegee acquired these subtypes through contact with these sites as drain samples routinely consisted of subtypes from both squeegee and pooled water samples. Pooled water and wheels also shared common subtypes illustrating the role of these sites in the dissemination of *Listeria* spp. It is possible that contamination patterns observed here resulted from microbial spread through the rinsing of, or direct contact with, the floor and/or pooled water, subsequently contaminating surfaces, including those in contact with food. Although exact contamination patterns cannot be determined, ribotype data suggests that the contamination of FCSs with *Listeria* spp., including *L. monocytogenes*, is typically preceded by contamination of NFCs reinforcing the role of environmental control in the prevention of food product contamination.

Our results indicate that the use of detection/isolation methods incorporating dual primary enrichment with a repair step allowing for the recovery of injured *Listeria* (mUSDA) enhances detection of *Listeria* spp., including *L. monocytogenes*. Furthermore, the addition of PCR to the mUSDA method increases sensitivity of detection while greatly reducing time to results. Results also suggest that the extent of farmstead cheese plant contamination with *Listeria* spp., notably *L. monocytogenes*, is comparatively low for dairy processing plants, especially those with contiguous farms. This study also illustrates the variation in environmental *Listeria* spp. contamination in farmstead cheese processing plants helping to identify points of control for use in designing more effective control and monitoring programs.

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Tables

Table. 1: Comparison of methods for the detection of *Listeria* spp. and *L. monocytogenes* from farmstead cheese processing plant environmental samples.

Method	<i>Listeria</i> spp.						<i>L. monocytogenes</i>					
	n	Sens. ^a	Spec. ^b	PV+ ^c	PV- ^d	Accuracy ^e	n	Sens. ^a	Spec. ^b	PV+ ^c	PV- ^d	Accuracy ^e
mUSDA	51	96.2	100	100	99.7	99.7	11	73.3	100	100	90.5	92.5
mUSDA/PCR	50	94.3	100	100	99.6	99.6	14	93.3	89.5	77.8	97.1	90.6
USDA	41	77.4	100	100	98.2	98.3	9	60	100	100	86.4	88.7

^a: Sensitivity (%) = 100* TP/(TP + FN)

^b: Specificity (%) = 100* TN/(TN + FP)

^c: Predicted value of positive test (%) = 100* TP/(TP + FP)

^d: Predicted value of a negative test (%) = 100* TN/(TN + FN)





























^e: Accuracy (%) = 100* TP + TN/(TP + TN + FP + FN)











Table. 2: *Listeria* spp. and *L. monocytogenes* isolation rates from environmental samples by individual farmstead cheese processing plant.

	Plant								
	A	B	C	D	E	F	G	H	I
Isolation rate (%)									
<i>Listeria</i> spp.	1.2	5.3	1.3	5.7	2.4	18.4	8.3	3.4	19.8
<i>L. monocytogenes</i>	0	0	0	1.1	0	10.3	4.8	0	1.2

Table. 3: Environmental *Listeria* spp. contamination patterns in farmstead cheese processing plants over multiple visits.

Plant	Visit(s)	Site	Ribotype ^a (lineage)	Riboprinter pattern
A	1	Floor (aging)	<i>Ls</i> DUP-1065	
B	1	Wheels (cart)	<i>Li</i> DUP-1005	
C	1	Drain (aging)	<i>Ls</i> DUP-1064	
D	1,2,3	Drain (prod.)	<i>Li</i> DUP-1019	
		Floor (entrance)	<i>Lm</i> DUP-1039C (II)	
	2		<i>Li</i> DUP-1009	
		Ladle	<i>Li</i> DUP-1006	
E	1	Floor (aging)	<i>Lw</i> DUP-1074	
	1	Floor squeegee	<i>Lw</i> DUP-1074	
F	1	Drain (aging room)	<i>Lm</i> DUP-1030B (II)	
	1,2,4	Drain (aging room)	<i>Lm</i> DUP-1042B (I)	
			<i>Li</i> DUP-1006	
	1,2	Drain (prod., R)	<i>Li</i> DUP-1006	
	1	Drain (prod., R)	<i>Li</i> DUP-1019	
	1,2	Drain (prod., L)	<i>Li</i> DUP-1006	
	2	Floor squeegee	<i>Lm</i> DUP-10144 (III)	
			<i>Lm</i> DUP-1030B (II)	
	2	Pooled water	<i>Lm</i> DUP-1042B (I)	
			<i>Li</i> DUP-1006	
	2	Hose nozzle	<i>Lm</i> DUP-1042B (I)	
			<i>Li</i> DUP-1006	
	2	Bucket to fill hoops	<i>Lm</i> DUP-1042B	
			<i>Lm</i> DUP-1030B (II)	
	3	Floor (prod.)	<i>Li</i> DUP-1006	

G	3	Floor (hallway)	<i>Lm</i> DUP-1042B (I)	
	3	Hose nozzle	<i>Li</i> DUP-1006	
	3	Wheels of cart	<i>Li</i> DUP-1006	
	3	Cut and wrap table	<i>Lm</i> DUP-1042B (I)	
	4	Drain (prod., L)	<i>Lm</i> DUP-1042B (I)	
			<i>Li</i> DUP 1009	
	4	Drain (aging room)	<i>Li</i> DUP-1010	
	4	Floor (hallway)	<i>Li</i> DUP-1006	
	4	Shoes	<i>Lm</i> DUP-1042B (I)	
	1	Pooled water	<i>Lm</i> DUP-1030B (II)	
	1	Drain (prod., 1)	<i>Lm</i> DUP-1030B (II)	
	2	Drain (prod., 1)	<i>Lm</i> DUP-1045A (II)	
	2	Drain (prod., 2)	<i>Lm</i> DUP-1030B (II)	
	2	Wall (near drain 1)	<i>Li</i> DUP-1005	
H	1	Drain (aging room)	<i>Li</i> DUP-1020	
	2	Floor (aging room)	<i>Ls</i> DUP-1064	
	2,3	Drain (aging room)	<i>Li</i> DUP-1005	
I	1	Drain (aging room)	<i>Li</i> DUP-1010	
			<i>Li</i> DUP-1005	
	1	Drain (other)	<i>Li</i> DUP-1005	
			<i>Li</i> DUP-1007	
	1,3	Drain (prod., R)	<i>Li</i> DUP-1006	
	1	Drain (prod., L)	<i>Li</i> DUP-1007	
	1	Floor (aging room)	<i>Li</i> DUP-1005	
	1	Floor squeegee	<i>Li</i> DUP-1007	
			<i>Li</i> DUP-1006	
	1,3	Floor squeegee	<i>Li</i> DUP-1005	
	2	Cut and wrap table	<i>Li</i> DUP-1005	

2	Pooled water	<i>Li</i> DUP-1009	
2	Cheese knife	<i>Li</i> DUP-1007	
		<i>Li</i> DUP-1006	
2	Drain (prod., L)	<i>Li</i> DUP-1009	
2	Floor (entrance)	<i>Li</i> DUP-1007	
3	Drain (prod., L)	<i>Li</i> DUP-1006	
		<i>Lm</i> DUP-1039C (II)	
3	Floor (entrance)	<i>Li</i> DUP-1005	
3	Hose nozzle	<i>Li</i> DUP-1006	
		<i>Li</i> DUP-1005	

^a: *Li* : *L. innocua*, *Ls*: *L. seeligeri*, *Lw*: *L. welshimeri*, *Lm*: *L. monocytogenes*

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